SYSTEMATIC APPROACH TO COMPARE THE INFLAMMATORY RESPONSE OF LIVER CELL CULTURE SYSTEMS EXPOSED TO SILVER, COPPER, AND NICKEL NANOPARTICLES

A Thesis

by

NIVEDITA BANERJEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Toxicology

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Approved by:

Chair of Committee,	Christie M. Sayes
Committee Members,	Stephen Safe
	Yanan Tian
	Robert Burghardt
Interdisciplinary Program Chair,	Weston Porter

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ABSTRACT

Systematic Approach to Compare the Inflammatory Response
of Liver Cell Culture Systems Exposed to Silver, Copper,
and Nickel Nanoparticles. (August 2010)
Nivedita Banerjee, B.S., University of Calcutta;
M.Sc., Bangalore University, India
Chair of Advisory Committee: Dr. Christie M. Sayes

Although nano-sized metal colloids are used in industrial and medicinal applications, little is known about the potential liver toxicity of these materials after occupational or intentional exposures. To begin to resolve some outstanding hepatotoxicity concerns, the inflammatory response of hepatocytes after exposure to metal colloids was assessed. Four ~30-nm-sized metal colloids, including silver (nano-Ag), copper (nano-Cu) and nickel (nano-Ni) were examined in an effort to understand the induced cytokine expression in a murine liver cell line (AML12). Here we also utilized another system, co-cultures of hepatocytes, Kupffer's cells, and lymphocytes isolated from C57BL6 mice. Cells were exposed to the materials over dose-response (0.1mg/L to 1000mg/L) and time-dependent (4 h, 48 h, and 1-week) studies. Cytotoxicity was measured via metabolism of resazurin and validated via MTT assay and cell counts. Inflammatory response was determined by cytokine profiles (TNF-α and IL-6), as

well as by mRNA and protein expression of heat shock protein (Hsp70). Results from cells exposed to nano-Ag to doses of up to 100mg/L exhibited no significant changes in cytotoxicity, IL-6, or TNF-α production, or Hsp70 expression. Both nano-Cu and nano-Ni exposed cells exhibited decreased metabolism, increased Hsp70 induction, and increased inflammatory responses (IL-6 and TNF-α). Dynamic light scattering and electron microscopy were used to characterize particle size and surface charge. All three metal colloidal systems demonstrated different particle size distributions, agglomerated sizes, and surface zeta potentials. Furthermore, each metal colloid system elicited different inflammatory biomarker responses and stress protein expression.

DEDICATION

To my parents

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Christie M. Sayes, and my committee members, Dr. Stephen Safe, Dr. Yanan Tian, and Dr. Robert Burghardt for their guidance and support throughout the course of this research.

I also thank Dr. Hansoo Kim and the Texas A&M University Microscopy and Imaging Center for help with transmission electron microcopy images of the nano-colloidal systems; Dr. Robert Taylor for his assistance with mass spectroscopy data; Dr. Emile A. Schweikert, Department of Chemistry, for assistance with SEM images; and also the Department of Veterinary Physiology & Pharmacology, College of Veterinary Medicine, Texas A&M University, for supporting this work.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, I would like to thank my mother and father for their encouragement and my husband for his patience.

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

1. INTRODUCTION

1.1 Industrial applications of metal nanoparticles

1.1.1 Nanoparticles

The word “nano” (derived from Greek word meaning extremely small) is a prefix indicating a measurement of one-billionth of a standard measurement (or 10^{-9} m). A nanoparticle is a cluster of atoms having an amorphous or crystalline structure, with at least one dimension measuring less than 100 nm. Due to their small size, these particles exhibit properties remarkably different from their bulk counterparts of identical chemical composition. Here, bulk counterpart refers to the micro (100–10,000 nm) or macro (>10,000 nm) size scale of the material with identical chemical, crystalline, and impurity composition when compared with the nanoparticle counterpart.

The unique set of physical and chemical properties (namely optical, electrical, magnetic, and catalytic properties) exhibited by nanoparticles has attracted universal interest among scientists and engineers to explore their use for promising applications such as drug delivery and novel composites. (Alivisatos, 1996; Crooks, 2001; Katz and Willner, 2004; Rosi and Mirkin, 2005). As the particle size shifts from the micro to the nano scale, the increased fraction of atoms at the surface of the particle allows interactions of the surface atoms to assume a larger role than that of interior atoms.

This thesis follows the style of Toxicology Letters.

The interactions of the surface atoms determine the characteristics of the particle.

As an illustration, a nanoparticle with a diameter a thousand times smaller than its micro-sized counterpart will also have approximately one-thousand-fold enhanced reactivity than the microparticle (Buzea et al., 2007). Accordingly, the ability to tune the particle size allows greater control of interfacial characteristic, such a reactivity. Additionally, quantum effects resulting from of a particle's discontinuous movements from quantum confinement distinguish nanoparticles from microparticles. These factors affect the particle surface reactivity, mechanical motions, optical and electrical properties, and magnetism (Buzea et al., 2007).

1.1.2 Metal nanoparticles

Metal nanoparticles possess outstanding optical and dielectric (high polarizability) properties. Metal nanoparticles have proven to be the primary building block of the next generation of multifunctional nanomaterials. These inorganic particles can be surface functionalized, chemically modified, and easily synthesized. There are various examples of metal nanoparticles, including silver, gold, copper, nickel, and zinc.

1.1.3 Types of metal nanoparticles

Metal-based nanoparticles can generally be classified in three types, as shown in Fig. 1

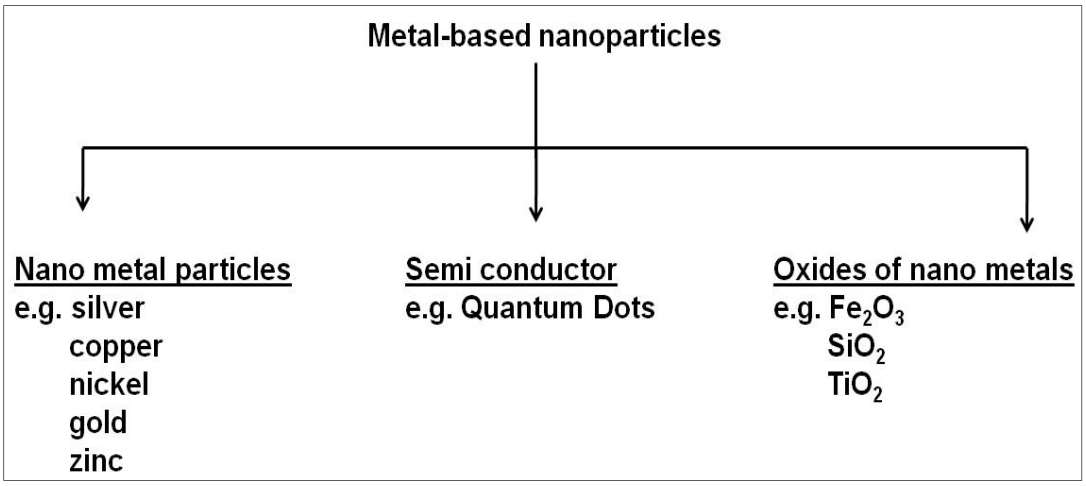

Fig. 1 Types of metal nanoparticles. Metal nanoparticles are include nano-sized metal particles, semiconductors, and oxides of nano-sized metal made of. Examples of nano metals particles are silver, copper, and nickel. Quantum dots are examples of semiconductor nanoparticles. Examples of metal oxide nanoparticles are iron oxide and titanium oxide.

One important application of iron nanoparticles is removing contaminants, such as chlorinated organic solvents and organochlorine pesticides, from groundwater, soil, and sediments. (Zhang, 2003; Nurmi, 2005). Iron nanoparticles are also used in sensor and transducer applications due to their magnetic properties. Sensors are devices which detect or quantitatively measure physical parameters, such as temperature and pressure. Transducers convert one form of energy to another form of energy; for example, a microphone converts sound energy into electrical signals. Magnetic nanoparticles have a number of biomedical applications, such as magnetic separation of biological materials (Kruis et al., 1998; Huber, 2005). The process of separating out specific biological entities from their native environment is facilitated by the

preparation of concentrated samples. This is achieved through a two-step process involving cell labeling with magnetic nanoparticles in first step and separating out the labeled cell using magnetic separation device in second step (Pankhurst, et al., 2009).

Zinc nanoparticles, having a high selectivity property, are used industrially as a catalyst. Selectivity property of a catalyst is the amount of reactant converted into useful product; in other words, a catalyst with good selectivity will maximize production of useful products and minimize production of undesired products. Zinc nanoparticles have high anti-corrosive, anti-bacterial, and anti-fungal properties useful in the coatings, bandages, plastic and textile industries (Revina, et al., 2007).

Gold nanoparticles, with unique plasmon-resonant optical scattering properties, are widely used in cellular imaging, drug delivery, and other biomedical applications (Wangoo et al., 2008). Surface plasmons are surface electromagnetic waves that propagate in a direction parallel to the metal surface. The excitation of surface plasmons by light is used to enhance surface sensitivity, such as that of fluorescence, for spectroscopic measurements. The most important and useful features of gold nanoparticles are lack of photobleaching, ease in synthesis, and stability in various suspension media (Chithrani et al., 2006; J. M. Klostranec and Chan, 2006; Chithrani and Chan, 2007). The image in Fig. , obtained from a transmission electron micrograph

(TEM), depicts the spherical shape of gold nanoparticles. Fig. 3 shows a schematic representation of the gold nanoparticle synthesis procedure.

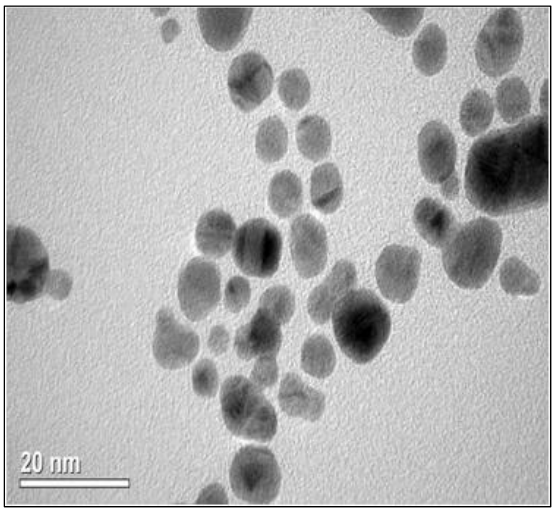

Fig. 2. Transmission electron microscopy image of gold nanoparticles. The point resolution is 2.7A and operation voltage is 200kV.The camera used was Gatan Tridiem GIF-CCD (2kx2k CCD camera). TEM grids were prepared by adding 2 µL of synthesized nanomaterial onto the copper grid surface (carbon-coated 400 mesh). (Image was taken by Banerjee and Sayes with the aid of Kim & Holzenburg, MIC, TAMU.)

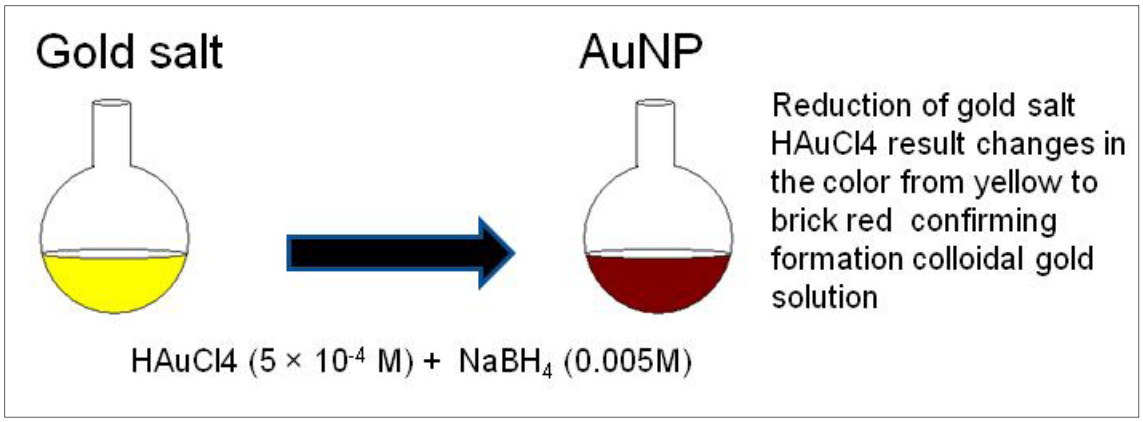

Fig. 3. Schematic representation of gold nanoparticle synthesis**.**

Another set of metal nanoparticles, quantum dots, are semiconductors exhibiting promising industrial uses, including LEDs (light emitting diodes), lasers, and telecommunication devices, such as optical amplifiers. Quantum dots are also known as nanocrystalline semiconductors. A semiconductor is a material with electrical conductivity between that of an insulator and a conductor. Modern day electronics are heavily dependent upon advanced semiconductors.

Through controlled tuning of the size and shape of a quantum dot, one can tailor the characteristics of a quantum dot for a desired application, such as a biosensor. Current biosensors use fluorescence-based dyes that limit them to a small number of colors as they emit light across a broad spectral width. In contrast, quantum dots can be fine tuned to emit at different wavelengths simply by altering the size of the dot (Wuister, et al., 2004). Electrical conductivity of a semiconductor can be influenced by an external stimulus, such as voltage or light. This property of semiconductors is used to advantage in electrical circuits and optical applications (EU-OSHA – European Agency for Safety and Health at Work. 2009). Fig. 4 shows quantum dots used as single electron transistor in various electronic industries (Michalet, et al., 2005; Xing, et al., 2009).

Metal oxide nanoparticles have proven to be useful for a variety of applications, including catalysis, sensors, and optical-electronics. Oxides of different metals, like titanium oxide (TiO_2) nanocrystal, are the main component of paint, pigments, and cosmetics. These particles can also be used to remove pollutants from air and water and sterilize polluted items (Kominami et al., 2000).

Iron oxide nanoparticles can be used for molecular imaging. Antibodies have been coupled with iron oxide nanoparticles and tested in vitro or in vivo (Kruis, et al., 1998; Huber, 2005). Zinc oxide nanoparticles have a unique set of mechanical and other physical properties. With the combination of a high melting point and high stability, zinc oxide nanoparticles have potential applications in various fields of modern technologies, such as in capacitors, photo-printing, and ceramic devices (Singbal, et al., 1997; Dem'yanets, et al., 2006). Transparent zinc oxide nanoparticles are used in the forestry and paper products industry as functional coatings to protect wood, paper, plastics, and textiles from ultraviolet radiation and microbial degradation.

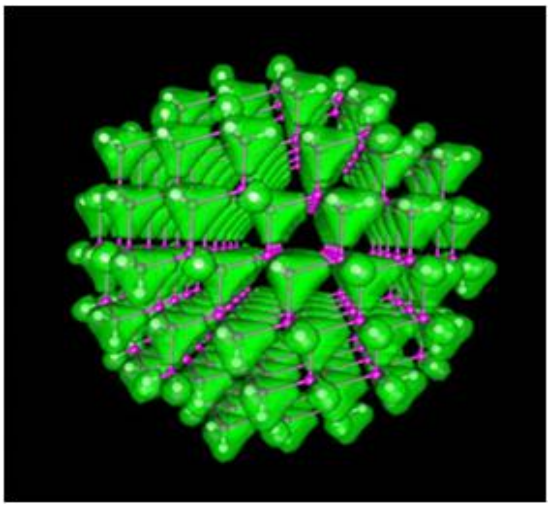

Fig. 4. Computerized representation of a GaAs quantum dot. Electron charge density (indicted by green) around atomic gallium nucleus (indicated by purple) for gallium arsenide containing 465 atoms (Cartoixa, X., and Wang, L.W., 2005.) (http://www.lbl.gov/Science-Articles/Archive/Quantum-Dot Electronics.html).

1.2 Mass-produced metal nanoparticles in industry

The increase in production of nanoparticles intended for different industrial and consumer applications also presents new risks and uncertainties to both human health and the environment. Growing mass production and increasing consumer use of nanoparticles result in a mounting number of workers and consumers exposed to nanoparticles. This leads to the need for an understanding of the possible fate, transport, and environmental effects on human health and the environment after exposure to nanoparticles. In our study, we have identified three different nanoparticles that have significant industrial applications.

1.2.1 Nanosilver

Silver nanoparticles have been proposed to be used as an antimicrobial, antibacterial, and antifungal agent (Antimicrobial agent is a general term for substances, chemicals, or drugs which either kill or reduces the growth of microorganisms such as bacteria and fungi) (Chen and Schluesener, 2008). Silver nanoparticles are extensively applied in medical applications like wound dressing, bandages, and in surgical tools. Surgical mesh coated with nanocyrstalline silver particles are used to reduce the prosthetic mesh infections after hernia and pelvic surgery (Arora et al., 2009). Silver nanoparticles, due to their antimicrobial properties, are used in household items like nanosilver-lined refrigerator seals, washing machine seals, washing detergents, and air-conditioner filters. Nanosilver particles are also used in clothing like footwear

and socks. They are also used in personal care products such as cosmetics, shampoos, and conditioners (EU-OSHA, 2009). Silver nanoparticles are also used in wastewater treatment and maintenance of indoor air quality (Chen and Schluesener, 2008). Due to high conductivity of silver nanoparticles, they can also be used as coatings in the form of glues, inks, paste and other polymers. Nanosilver particles are currently used to make thinner pastes and coatings as opposed to what was previously made from silver powder (EU-OSHA, 2009).

1.2.2 Nanocopper

Copper nanoparticles are used in industry in coatings to reduce growth of living organisms like fungi (Cioffi et al., 2005; Lei et al., 2008) and as a highly reactive catalyst in organic reactions. Due to their excellent mending properties, copper nanoparticles can be used as additives in lubricants, polymers, and plastics to reduce friction. (Liu, et al., 2004). Studies have also shown to improve charge-discharge properties, copper nanoparticles deposited onto graphite can be utilized (Guo, 2002; Chen et al., 2006). Copper nanoparticles is also known to inhibit the growth of microorganisms (Cioffi et al., 2005). Copper nanoparticles are also used as additives in poultry and livestock feed (Lei et al., 2008).

1.2.3 Nanonickel

Nickel nanoparticles are widely used in industry due to their high surface energy, high surface area, high magnetism, and low melting and boiling points. Nickel nanoparticles are used to improve the wear-resistance and high-temperature oxidation resistance of titanium and titanium alloys (Yi, et al., 2007). Nickel nanoparticles are also used in fuel cell electrodes due to their increased surface area and activity. The enhancement of magnetic, anti-corrosive, antimicrobial, and durability properties of the material is observed in coatings made of nickel nanoparticles. Nickel nanoparticles are also used in paints and as inks. Also, it has been reported that nickel nanoparticles are used in various filtration techniques such as gas separation, bacteria and biohazard removal, and in air purification.

1.3 Occupational-based exposure and the field of exposure science

Recently, there has been a rapid international development of companies, such as Auterra (previously Applied Nanoworks), PlasmaChem, OceanNanotech and Nanotherapeutics, producing nanoparticles and nanoparticle-based consumer products. It has been estimated that by 2020, roughly 2 million workers will be employed in nanotechnology based industries (Schulte, et al., 2008). These developments have drawn attention of researchers to study the potential health risks of workers who might be unintentionally exposed to those nano-sized particles. Inhalation and trans-dermal absorption (Paul, et al., 2006) can be classified as two types of occupational-based exposures. There is limited

epidemiological data available on occupational exposure to nanoparticles, which is used in exposure science. Exposure science is the study of interaction of chemical, physical or biological agents present in the environment with human body. Exposure science also advances knowledge of the mechanisms and dynamics of events either causing or preventing adverse health effects.

1.3.1 Examples of occupational exposure

Several scientists are currently working on the benefits of nanoparticle-based products. Workers in nanoparticle-based industries are expected to be in direct contact with metal nanoparticles. Some of these nanoparticles have been proven to be toxic at certain exposure levels (Braydich-Stolle et al., 2005; Hussain et al., 2005; Chen et al., 2006; Hussain et al., 2006). It is expected that nanoparticles might become part of airborne dust if an accidental release takes places at a workplace. Dispersion of an accidentally released stream containing nanoparticles might pose a health risk to plant personnel based on level of exposure. Moreover, as industrial pollution increases with time, the concentration of nanoparticles in the air is also expected to increase. A clear understanding of the toxicity of these nanoparticles is therefore required to define the safe levels of nanoparticle exposure.

1.3.1.1 Occupational exposure and exposure assessment of various toxic compounds and substances

Working with toxic materials is expected to result in chronic occupational exposure of the personnel working and handling the materials. As an elaborate

example, occupational exposure to ethanol is described below to emphasize that similar effect can be expected from emerging nanotechnology based industries.

Industrial ethanol exposure: Industrial ethanol is currently one of the largest volume organic chemicals produced. It is widely used in industry to manufacture different types of consumer products. Occupational exposure to ethanol mostly occurs through inhalation and dermal routes. The use of ethanol-based products, such as antiseptic hand gels which is used as antibacterial agent to combat the spread of antibiotic-resistant bacterial strains, may also result in increased dermal exposure for both workers in industries and scientists in the laboratory and can act as a potential cancer risk (Bevan et al., 2009).

Nanoparticles exposure: Personnel working in nanoparticle-based industries are exposed to different types of nanoparticles. As an example, a number of studies showed exposure to carbon black can lead to adverse effects in humans (Guo et al., 2009). It is hence important to understand and assess the risk associated with nanoparticle exposure. Risk assessment is the basis of assessing and regulating nanomaterials to protect health and the environment. Biological markers such as urine or blood are used to assess the exposure to toxic metal or chemicals. Urine can be more conveniently collected than blood. Urine is the most reliable and preferred indicator, as the kidney is the main nexus of elimination of toxic metals (e.g., zinc oxide nanoparticles, inorganic arsenic) or other chemicals through excretion. In addition hair is used for

forensic investigation of occupational exposure of toxic metal (Farmer and Johnson,1990).

Occupational exposure limit values (OELs): OEL for hazardous materials is an important tool for health risk assessment. It also helps in managing valuable information for occupational safety and health activities concerning hazardous substances. The National Institute for Occupational Safety and Health (NIOSH) is the primary federal agency conducting such research and provides guidance on the occupational safety and health implications and applications of nanotechnology.

Current safety practices: To minimize consequences from occupational exposure hazard, Occupational Safety and Health Administration (OSHA), has formulated safety standards to be followed in industries. To maintain safe working conditions and to prevent injury to personnel, OSHA standard 1910.9(a) and 1910.9(b) of 29CFR requires:

- Personal protective equipment (PPE): Standards in this part requires the employer to provide personal protective equipment (PPE), including respirators and other types of PPE, because of hazards to employees impose a separate compliance duty with respect to each employee covered by the requirement.
- Training: Standard in this part requires training on hazards and related matters should be provided to employees, or employers should institute or implement a training program for the same.

However, these OSHA standards may not address safety issues arising from potential nanoparticles exposure. Although the potential health effect of such exposure is not fully understood at this moment, OSHA, as part of a government-wide coordination effort, OSHA is working with other federal agencies like NIOSH to address issues related to the impact of nanomaterials on human health and the environment. Current OSHA standards which may be applicable to nanoparticle exposure in workplace are 1904 (Reporting occupational injuries), 1910.132 (PPE), 1910.133 (Eye and face protection), 1910.134 (Respiratory protection), 1910.138 (Hand protection), 1910.141 (Sanitation), 1910.1200 (Hazard communication) and 1910.1450 (Occupational exposure to hazardous chemicals in laboratory).

While some nanoparticles like titanium dioxide, carbon black, or nickel nanoparticles are handled both in industry and in the laboratory, there are newly synthesized nanoparticles which are manufactured and handled only at the laboratory scale. Toxicity of such particles is not well understood. Students or research scientists can be affected while working with these nanoparticles. To improve the working condition in laboratory some generic precaution should be taken derived from general safety practices:

- nanoparticle work should be performed in a low velocity, laminar– flow type hood (e.g. Flow Science) to avoid inhalation of aerosols containing nanoparticles;

- protective cloths like aprons and gloves should be used to avoid contact of nanoparticles with the bare skin;
- safety goggles should be worn to prevent eye exposure to nanoparticles;
- in some cases, appropriate nasal masks should be used while working with nanoparticles in the laboratory.

1.3.2 Unintentional exposures through inhalation

Effects on health through unintentional inhalation exposure are a major concern. At the workplace, employees are likely to be exposed to nanoparticles during the production process, testing of products, transport, storage, or waste removal/treatment. Any accident occurring due to a system failure (or other reason) has the potential of releasing nanoparticles in large amounts, endangering not only the working personnel but also the neighboring people. Airborne nanoparticles can be easily dispersed and spread in environment (Oberdörster et. al. , 2005)

1.3.3 Unintentional exposures through trans-dermal absorption

Another important route of unintentional exposure in the workplace is through transdermal absorption. The nanoparticles present in the environment could be in contact with exposed skin. Because of their small size, nanoparticles have been shown to penetrate clothing and translocate through the dermal and subcutaneous layers of human and animal skin (Monteiro-Riviere, et al., 1990; Scott et al., 1991; Oberdörster et. al., 2005).

1.3.4 Consumer-based exposure

In addition to unintentional exposure, exposure to nanoparticles can also occur through the usage of the nanoparticle-enabled products. Nanoparticles are used in personal care products, such as cosmetics and sunscreens (Colvin, 2003). The use of these engineered nanoparticle-based products can lead to direct dermal exposure. Typically, consumer-based exposure routes can be further classified into two specific routes, namely ingestion and transdermal absorption.

1.3.4.1 Intentional exposure through ingestion

Due to the small particle size and the increase in nano-enabled devices, it is expected that nanoparticles will be found in the environment, including soil, water, and the food chain. Thus, increasing the likelihood of trace amounts of nanoparticles along with food and drinking water. In addition, groundwater remediation releases nanoparticles into the environment, soil, and water that may lead to direct human exposure (Boxall et al., 2007). The main likely route of entry of nanoparticles to the intestinal tract is through consumption of nano-sized food and drink supplements or via additives, such as metals, minerals, clays, or lipids. The potential effects of the nanoparticles on ecosystems depend not only on the nanoparticle characteristics but also on the amount of nanoparticles emitted by industry**.** Various companies are conducting research on nanoparticle-based food packaging (Boxall et al., 2007; Chaudhry et. al. 2008).

1.3.5 Intentional exposure through transdermal absorption

The use of nanoparticle-based products (such as cosmetics, sunscreen, and other creams) can cause direct dermal exposure that is classified as an intentional exposure. The amount of exposure depends on the concentration of the nanoparticles in the cosmetic product and the frequency of use by the consumer. Sunscreen-grade nanoparticles mainly consists of titanium dioxide (TiO_2) and zinc oxide (ZnO), which are used to reflect and scatter the UV light (Popov, et al., 2005; Nohynek, et al., 2008). ZnO and TiO_2 are also used extensively in other cosmetic products, such as liquid and powder foundations, eye shadow, and blush (Nohynek, et al., 2008). The availability of nanoparticle-based consumer products is increasing with time and is readily available in today's market.

1.4 Nanoparticles exposure to liver

1.4.1 Introduction to the concepts of absorption, distribution, metabolism and excretion

The engineered metal nanoparticles are subjected to a pre-clinical test process like absorption, distribution, metabolism and excretion (ADME) before their use in biomedical applications. The factors that control the ADME profile are not well understood.

1.4.1.1 ADME definition

Absorption, distribution, metabolism and excretion influence the kinetics of drug distribution throughout the tissue and thus play an important role in pharmacology study.

Absorption: The first step of ADME controls the bioavailability of compounds. The compounds first enter the blood through the process of absorption. Subsequently the compounds are taken up by the targeted tissue or cells.

Distribution: In the next step, the absorbed compound is carried to the effector's site. Most often this happens via the blood. The flow of blood facilitates the distribution of the compound to different organs and tissues.

Metabolism: The majority of the compounds metabolism is carried out in the liver by CYP450 enzymes. During the process, the parent compound is converted into its metabolites. In general these metabolites are pharmacologically inert in nature than the parent compound.

Excretion: The compounds along with their metabolites are removed from the body through the process called excretion. The organ involved in this step is liver. The accumulation of these compounds in the body can have adverse effects on metabolism.

1.4.2 Linking ADME to the liver

Exposure to nanoparticles can occur through water, food, cosmetics or drugs. The nanoparticles are able to enter the body through the inhalation

process, trans-dermal absorption or ingestion. These are also known to be potential portals of entry. Recently it has been shown that some of the metal nanoparticles clearly accumulate in different organs, such as liver, kidney and lungs (Kim et al., 2008). The nanoparticles are taken up into the targeted tissue or cells after distribution to different organs. Ultimately, the particulate matter that enters into the body might be metabolized and excreted through the liver (Wan et al., 1991; Pinheiro et al., 1996; Das and Buchner, 2007; Medina et al., 2007). The hazardous nanoparticles are either excreted from the different organs or persist inside the body. To correlate the broad concept of ADME with toxicity, it is very important to understand the potential of a compound in exerting harmful effect on the organs. Nanoparticles are taken up into the cells through the process of absorption and distribution. Once the nanoparticles are absorbed and distributed into the cells, they come directly in contact with liver during metabolism. In the process, this might lead to liver toxicity.

1.5 Liver cell toxicity

The liver is the largest gland in the body. It is located below the diaphragm and is anterior to the stomach. It consists of four lobes: the right lobe is the largest and is far larger than the remaining three; the left lobe and two other smaller lobes are placed behind the right lobe. The lobes of liver are shown in Fig. 5. To understand the function and dysfunction of the liver it is very important to know the liver structure. In this section, the following topics are discussed:

1. Structure of the liver
    a. The hepatic vascular system
    b. The biliary system,
    c. The different cell types of the liver
    d. Interaction between hepatocytes and non parenchymal cells
    e. Importance of co-culture system
2. Physiological function
3. Drug-induced hepatotoxicity
4. Biomarkers for liver damage

1.5.1 Structure of the liver

The liver is composed of numerous hexagonal lobules oriented around a central vein. These hexagonal lobules, known as hepatic lobules, are considered to be the structural unit of the liver. Portal triads located at the corners of each hepatic lobule contain the branch of portal vein, a hepatic arteriole and a bile duct. In Fig. 6, a portal vein, a hepatic arteriole, and a bile duct are illustrated in red, blue and green dots. Each hepatic lobule can be divided into three regions namely centrolobular, midzonal, and periportal. The blood enters the portal triads through the portal vein and hepatic artery.

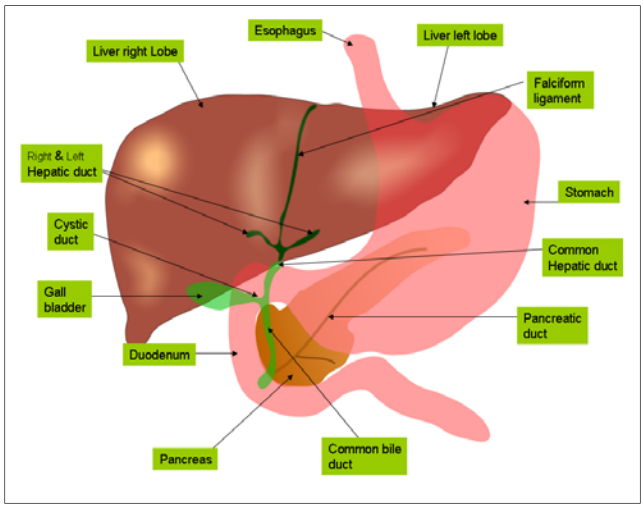

Fig. 5. Liver structure.. (Punnoose, J.K. 2007.)

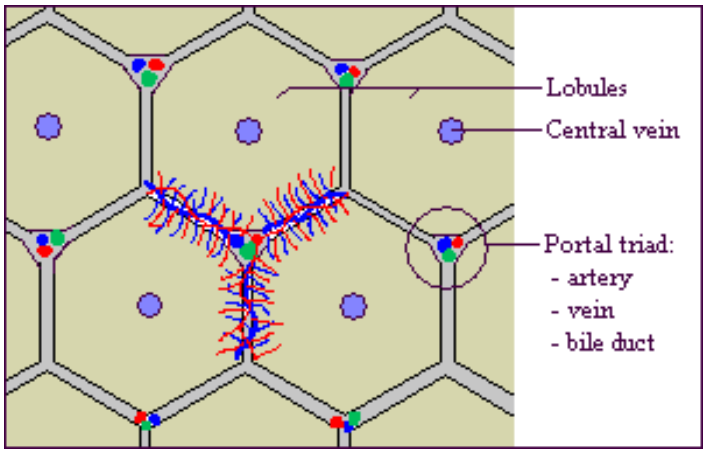

Fig. 6. Hepatic lobules showing portal triads containing branch of artery, vein, and bile duct at the corner and central vein at the center of each lobule. (Bowen, R., 2004.)

1.5.1.1 The hepatic vascular system

Within a liver, 75% of the blood flows in the portal vein and is classified as venous blood**.** The venous blood, returning from small intestine, stomach, pancreas, and spleen, ingresses into the portal vein located in the portal triads of the hepatic lobule. The remaining 25% of the blood in liver flows through hepatic artery and is known as arterial blood. The blood entering the portal triad through hepatic artery and portal vein is mixed as blood enters sinusoids in the liver. Sinusoidal blood vessels have a fenestrated epithelium. The blood mixes thoroughly along the cords of hepatocytes and finally flows into terminal hepatic venules.

1.5.1.2 The biliary system

The biliary system is a series of channels and ducts that conveys bile - a secretory and excretory product of hepatocytes - from the liver into the lumen of the small intestine. Hepatocytes secrete bile into the canaliculi, and those secretions flow parallel to the sinusoids, but in the opposite direction that blood flows. A scehamatic diagram of liver biliary system is shown in Fig. 7.

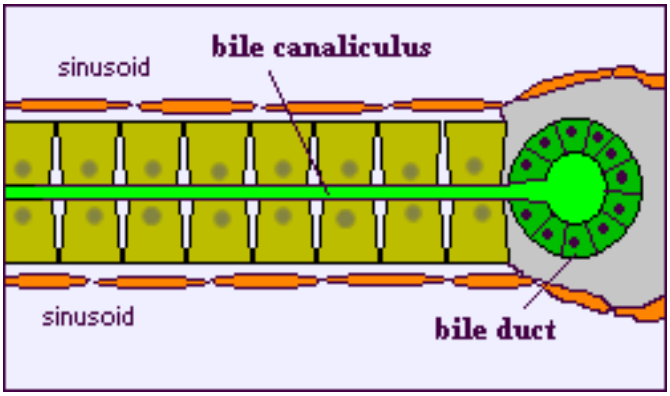

Fig. 7. The biliary system showing hepatocytes secreting bile into the canaliculi. Bile secretion flow is in the opposite direction than the blood flow. (Bowen, R., 2004.)

1.5.1.3 Parenchymal and non-parenchymal liver cells (hepatocytes)

Of the liver's cytoplasmic mass, 70 to 80% is composed of hepatocytes. The remaining part of the liver is composed of non parenchymal cells. The hepatocytes cells are polygonal and their sides can be facing either the sinusoid (sinusoidal face) or neighboring hepatocytes (lateral faces).

Heopatocytes are epithelial cells which contain the major enzymes for drug and xenobiotic biotransformation. These include the major phase I drug metabolism enzyme family of P450 and phase II enzymes such as UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), *N*-acetyltransferase (NAT), and glutathione *S*-transferase (GST). Hepatocytes also contain all the gene regulation pathways for P450 induction.

1.5.1.4 Liver non-parenchymal cells (sinusoidal endothelial cells, Kupffer's cells and hepatic stellate cells)

*Hepatic sinusoids (sinusoidal endothelial cells)***:** These are larger capillaries which form channels between cords of hepatocytes. The blood percolates in the sinusoidal channels before it exits through the terminal vein. Sinusoids serve hepatocytes with the oxygen-rich blood from hepatic artery and the nutrient-rich blood from the portal vein.

*Kupffer's cells***:** These are the specialized resident macrophages of liver located in the lumen of the sinusoids. They compose 80% of the fixed macrophages of the body. Kupffer's cells are developed in the bone marrow. The main function of Kupffer's cells is to degrade ingested particulate matters.. Kupffer's cells synthesize several mediators, like cytokines, chemokines and help strengthen the immune response in the liver (Ramadori and Armbrust, 2001). Kupffer's cells are source of cytokines as shown in Fig. 8.

*Hepatic stellate cells***:** Ito cells are also called fat-storing cells or hepatic stellate cells. These cells are located in the space of Disse between endothelial cells and the cord of hepatocytes. Ito cells synthesize collagen as demonstrated in Fig. 8 and are the major site for vitamin A storage in the body. In addition, there are some pit cells and a lymphocyte-type cell. The pit cells are hepatic natural killer cells (lymphocytes) that are located in the liver sinusoids.

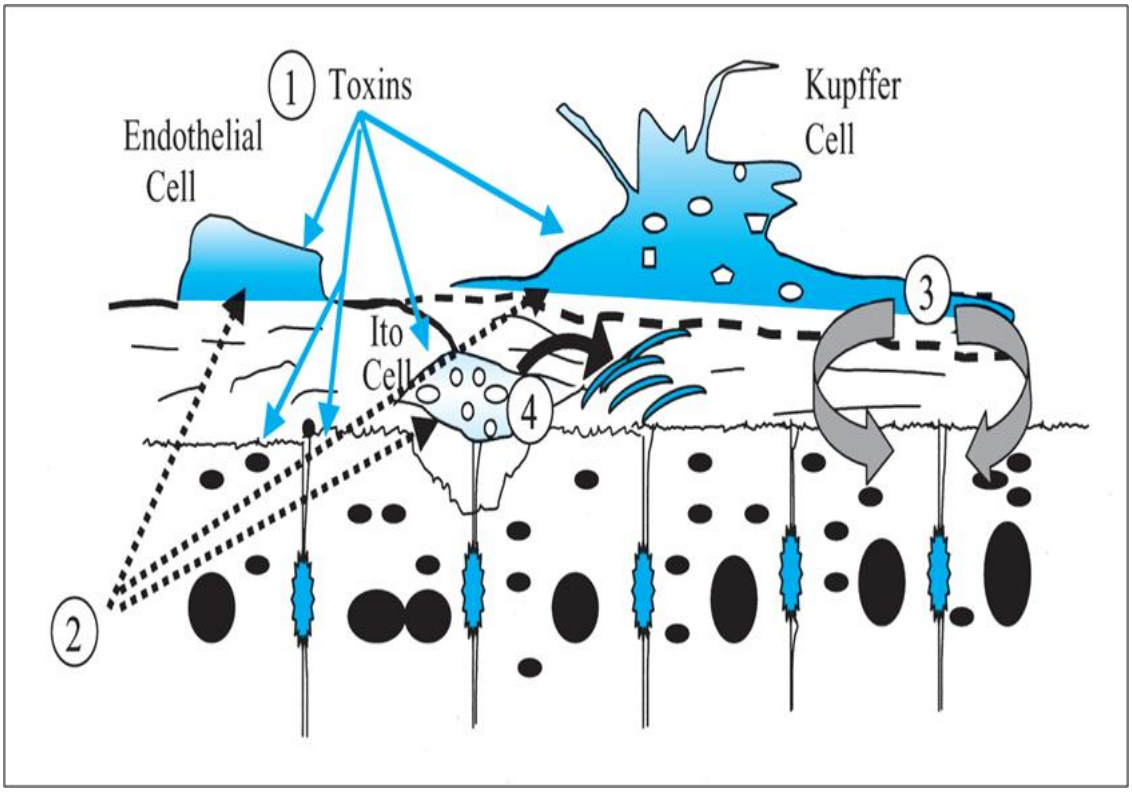

Fig. 8. Schematic of the complex cascade of toxin-evoked interactions between hepatocytes and sinusoidal cells. Steps of creation of a hepatotoxicity scenario could involve: (1) Toxin causing injury to hepatocytes; (2) Signals from the injured hepatocyte to Kupffer and Ito cells as a result of step (1); (3) Kupffer cell releases cytotoxins and (4) Collagen secretion from Ito cells.(Klaassen,2007)

1.5.1.4 Interaction between hepatocytes and NPC (Kupffer's cells and Ito cells) in response to toxin

Kupffer cells and Ito cells showed an activated morphology after exposure to hepatotoxicants, as show in Fig. 8. Activated Kupffer cells secrete large amounts of soluble cytotoxins. Upon activation Ito cells secrets appreciable amount of collagen. Ito and kupffer's cells after activation increase in number.

Hepatoxicity and injury due to interaction of many toxins results in exchange of signals between toxicant and activated Kupffer cells.

1.5.2 Physiological functions of the liver

The liver regulates most chemical levels in the blood and excretes a product called bile, which helps carry away waste products from the liver. All the blood leaving the stomach and intestines passes through the liver. The liver processes this blood and breaks down the nutrients and drugs into forms that are easier to use for the rest of the body. Some of the functions of liver are as follows:

- carbohydrate, protein and fat metabolism;
- production of bile, which helps carry away waste and break down fats in the small intestine during digestion;
- production of cholesterol and help to carry fats through the body;
- conversion of excess glucose into glycogen for storage (glycogen can later be converted back to glucose for energy);
- processing of hemoglobin for use of its iron content;
- conversion of poisonous ammonia to urea (urea is an end product of protein metabolism and is excreted in the urine);
- clearing the blood of drugs and other poisonous substances;
- blood clotting;
- resisting infections by producing immune factors and removing bacteria from the blood;

- filter the harmful substance from the blood, such as alcohol;
- storage of vitamins and minerals (Vitamins A,D, K, and B12)

After the liver has broken down harmful substances, liver by-products are excreted into the bile or blood. Bile by-products enter the intestine and ultimately leave the body in the form of feces. Blood by-products are filtered out by the kidneys, and leave the body in the form of urine.

Hepatocytes house many metabolic pathways and are comprised of various enzymes which play important role during the process of metabolism. The liver is extremely active in oxidizing triglycerides to produce energy. The bulk of lipoproteins are synthesized in the liver. The liver synthesizes large quantities of cholesterol and phospholipids. Some of these arestored with lipoproteins and made available to the rest of the body. The rest is excreted in bile as cholesterol or excreted after conversion to bile acids. Hepatocytes are also responsible for synthesis of most of the plasma proteins. Albumin, the major plasma protein, is synthesized mostly by the liver.

1.5.3 Drug-induced hepatotoxicity

Most drug-induced hepatotoxicity is not clearly understood (Srivastava, et al., 2010). As the liver is the principal site for drug metabolism, it is known to be the target organ for drug-induced toxicity. Drugs that are taken up inside the body are generally converted to biologically inactive forms and are eliminated out through excretion. But some of these drugs undergo biotransformation and are converted to active metabolites. These metabolites can interfere with cellular

functions and can lead to hepatoxicity (Lei et al., 2008; Mil'to and Dziuman, 2009; Srivastava, et al., 2010).

The liver is exposed to drugs and xenobiotics immediately after absorption of drugs from gastro-intestinal tract. Once the liver is exposed to drugs, the phase I and phase II enzymes present in the liver control the biotransfromation of lipophilic compounds into water soluble derivatives and are readily removed from the body through excretion. But certain drugs, such as acetaminophen (APAP) undergo biotransformation to form toxic or reactive metabolites (Keisu and Andersson, 2010 ; Srivastava, et al., 2010). These toxic and reactive metabolites can interact with the cellular components like cellular protein, lipids or nucleic acids and interfere with the cellular functions like protein dysfunction, lipid peroxidation, DNA damage, oxidative stress or mitochondrial dysfunction. The toxic metabolites can also cause hepatocyte death, sinusoidal disorder or bile duct damage (Keisu and Andersson, 2010; Srivastava, et al., 2010), The schematic representation of drug induced hepatotoxicity is shown in Fig. 9.

1.5.4 Biomarkers for liver damage

During clinical trials it is very important to monitor hepatic laboratory parameters, such as serum aminotransferases (ALT; AST) and to investigate their potential clinical significance as indicators of hepatotoxicity (Keisu and Andersson, 2010). Alanine aminotranferease (ALT) and aspartate aminotransferase (AST) are enzymes located in liver cells that are released into

the general circulation (blood) when liver cells are injured. Both ALT and ALT can be measured in blood as a sensitive indicator of liver cell damage and liver injury.

ALT is found in large amounts in the liver. It is also found in the kidneys, heart, and skeletal muscle. AST is found in heart, kidney, and liver tissue. As a result of injury, the level of ALT and AST serum biomarker are increased. It must be noted that the ALT is a more specific indicator of liver inflammation than the AST.

1.6. Liver cell toxicity from metal ions

Metal nanoparticles are composed of metal ions. In a solution containing metal nanoparticles, the pH can vary over time, thus leading to dissociation of metal particles into metal ions. In such an instance, metal nanoparticles intended to act as particles might induce toxic effects through the release of metal ions.

1.6.1 Silver nanoparticles dissociate into silver ions

In the environment (i.e. air, soil, and water), silver nanoparticles do not remain in the nano-size regime. They can agglomerate to form larger clusters of silver particles. The formation of silver particle clusters reduces the surface area-to-volume ratio. As a result, these clusters are less effective as an antimicrobial agent (Monteiro, et al., 2009).

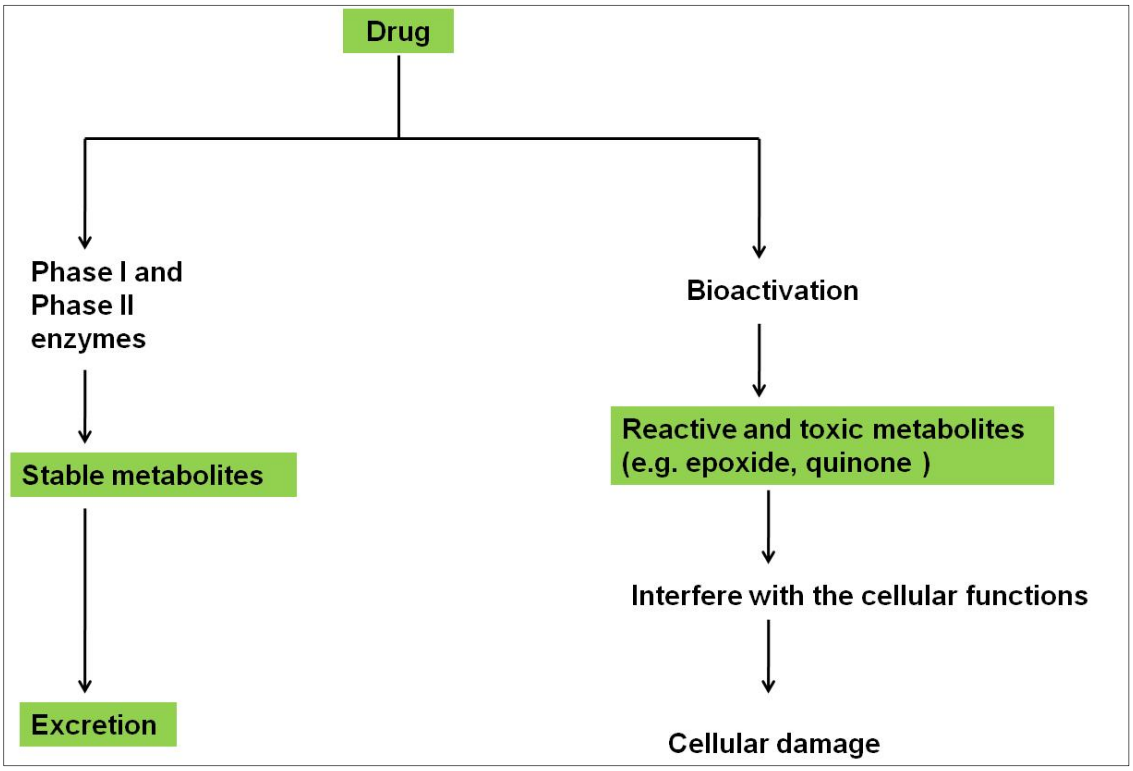

Fig. 9. Schematic representation showing relationship between drug metabolism and toxicity. Accumulation of parent drug or formation of a reactive/ toxic metabolite that cannot be detoxified can cause toxicity. Epoxides or quinone are the reactive and toxic metabolites that interact with different macromolecules (such as protein, nucleic acid) and thus interfere with the cellular function. (An epoxide is a cyclic ether with three ring atoms. A quinone is a class of organic compound derived from aromatic compounds such as benzene by exchanging an even number of –CH= groups by –C(=O)– groups, with any necessary rearrangement of double bonds).

Additionally, in this clustered form, silver particle agglomerates are non-ionic and relatively inert to the environment and aquatic life. In the presence of a strong oxidizing agent (such as halides), however, silver nanoparticles can easily dissociate into silver ions. Numerous studies in literature have shown the release of silver ions from silver nanoparticles (Monteiro-Riviere, et al., 1990; Choi et al., 2008; Kong and Jang, 2008; Brandenberger, et al., 2010).

Nanocomposites (a composite material one of whose constituents has at least one dimension less than 100 nm) made from silver nanoparticles in a polyamide matrix have shown evidence of silver ion release through diffusion (Damm and Münstedt, 2008).

1.6.1.1 Interaction of silver ions with micro-organisms

Silver ions play an important role as an effective antimicrobial agent (Lok, et al., 2006). Silver ions can kill or slow down the growth of a wide variety of yeast, fungi, viruses, and bacteria (Lansdown, 2006). According to some literature reviews (Hussain et al., 2005; Shin et al., 2007), free silver ions have two antimicrobial mechanisms. Firstly, presence of silver ions breaks the disulfide bonds of the bacterial proteins, an essential bacterial structure. Secondly, silver ions produce reactive oxygen species which kills the microorganism. Silver ions also inhibit cell cycle and cause cell death in bacteria (Bicanova and Thinschmidt, 1985; Lansdown, 2007; Shin et al., 2007; Chen and Schluesener, 2008).

1.6.1.2 Interaction of silver ions with mammalian cells

Some silver containing substances are believed to be beneficial to human/animal health. Mammalian cells are eukaryotic and hence display a strong unsusceptibility to the effects of silver, therefore allowing the use of silver in treating human disease (Goodman and Gilman, 1970). Human health care providers have used silver for diverse purposes for several thousand years (Bicanova and Thinschmidt, 1985). Thus, silver nanoparticles are extensively

applied in medical instruments and wound dressings (Arora et al., 2009). The use of silver nanoparticles in wastewater treatment and maintenance of indoor air quality is also found in literature (Chen and Schluesener, 2008). Today, forms of silver are found in functional products for water purification, bandages, algaecides (for pool water), and integrated into fabrics for medicinal benefits. Recently various clinical trials have been performed with silver nanoparticles. Examples include application of silver nanoparticles in acne treatment, oral rinses, and toothpaste. Although silver nanoparticles possess a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria and fungi, silver nanoparticles are less toxic to mammalian cells (Arora et al., 2009). As a result, silver nanoparticles are used in various medical devices (Monteiro, et al., 2009).

1.6.2 Copper nanoparticles dissociate into copper ions

Very few studies have been performed to estimate the release of copper ions from copper nanoparticles. Studies have shown that copper nanoparticles can be effective vehicles for copper ion delivery to *in vitro* cultures (Kothapalli, and Ramamurthi, 2009).

1.6.2.1 Interaction of copper ions with micro-organisms

Nano-sized copper particles are also known to inhibit the growth of micro-organisms (Cioffi et al., 2005). Copper metal reacts with proteins of microorganism by combining the -SH groups of enzymes, and this reaction leads

to the inactivation of the microorganism proteins. Copper nanoparticles were also used as an antimicrobial agent against E. coli and B. subtilis (Yoon, et al., 2007).

1.6.2.2 Interaction of copper ions with mammalian cells

However, if copper intake exceeds the human tolerance level, it may lead to toxicity, high blood pressure, several mucosal irritations, hepatocellular necrosis, changes in lipid profiles, capillary damage oxidative stress and can even lead to death. (Zhou et al., 2009). Copper nanoparticle exhibits quite different sets of toxicological effects compared to its bulk counterpart (Chen et al., 2006; Warheit et al., 2007). The potential risks of nano-Cu particles on human health have been shown in various studies but the mechanism of toxicity has not been reported (Lei et al., 2008).

There are several studies showing the toxicity of copper compounds. The target organs for the copper toxicity are liver and kidney (Lei et al., 2008). The damages in the liver and kidney are observed in rats exposed to a high dose of copper nanoparticles. As marker of liver damage there is an increase in enzyme aspirate aminotransferase (AST) and alanine aminotransferase (ALT) level in serum. Additionally renal glomerulus dysfunction and loss of kidney weight are also observed (Lei et al., 2008) due to nano copper toxicity. Recently, various proteomics and genomic studies have been widely applied in toxicological research to better understand the toxicity of copper nanoparticles.

1.6.3 Nickel nanoparticles dissociate into nickel ions

Very few works demonstrating liberation of Ni ion from Ni nanoparticles or from Ni compound-based nanoparticles exist. While studying the stability of Ni nanoparticles, (Lefondeur, et. al., 2001) observed increase of Ni^{+} concentration by evaluating EPR spectra. Electron paramagnetic resonance spectroscopy is a technique for studying chemical species with one or more unpaired electrons. The observation was attributed to the partial decomposition of the NiRCS compound (Ni nanoparticles inserted in an organic matrix, supported on γ alumina), which acted as a catalyst in a benzene-hydrogenation reaction.

1.6.3.1 Interaction of nickel ions with micro-organisms

Nickel compounds (nanoNi and nickel oxides) have extensive industrial applications and are investigated as an antimicrobial agent (Pang, et al., 2009). Nickel nanoparticle coating shows photocatalytic properties and can be used as an antimicrobial agent (Rana, et al., 2005). Nickel is an essential nutrient for select microorganisms (Mulrooney, and Hausinger 2003). These select microbes are capable of sensing Ni ion concentration in a cell. However, precise mechanism of interaction of these microbes with Ni ion is still unknown and requires further investigation (Mulrooney, and Hausinger 2003). Effect of nickel ion concentrations on microbial populations has also been studied (Ashley, 1982). The authors attributed starch hydrolysis to increased nickel ion concentration. The dissolved nickel was found to be hostile to the methanogenic process.

1.6.3.2 Interaction of nickel ions with mammalian cells

Nickel compounds (nano-Ni and nickel oxides) are known to be very toxic nanoparticles. Toxicity caused by nickel is dependent on route of exposure and also on metabolism of these nickel particles. A number of studies have shown hepatic toxicity associated with nickel exposure (Coogan et al., 1989). Metallic nickel nanoparticles cause higher cytotoxicity and apoptosis than its micro size counterpart (Zhao et al., 2009).

Consumption of nickel compounds leads to environmental and human health hazards. Metallic nickel particles are known to cause apoptosis, but the mechanism of the cell death induced by this metallic particle is yet to be understood (Zhao et al., 2009). The toxicological effect of nickel nanoparticles and metallic nickel is shown to be significantly dependent on the size of the particles (Zhao et al., 2009).

2. A SYSTEMATIC APPROACH TO COMPARE THE INFLAMMATORY RESPONSE TO INDUCED NANO-SIZED METAL COLLOIDS

2.1 Introduction

Research and development efforts in metal and metal oxide nanomaterials are providing new opportunities for drug delivery, food packaging, cosmetic, and electronic applications that were not possible a few years ago (DeWild et al., 2003). However, along with the unique applications of nano-sized metals there are also unique cellular interactions at the nano-bio interface due to the particle size. Furthermore, because metal materials readily equilibrate in the aqueous phase by losing electrons to form cations, nano-sized metal colloids could be highly reactive within the engineered application, and could therefore pose a potential risk to human health and the environment (Colvin, 2003; Carlson et al., 2008).

Nano-sized metal colloids possess larger surface area and surface free energy when compared to micron-scale metals, which may lead to either a simulated metal ion response by an intact nanoparticle or an increased dissociation of cations from the nanoparticle surface into the surrounding matrix

(i.e. water, biological fluid, or cells). Some toxicological evaluations of nano-sized metal colloids have shown that as nanomaterial dose increases, the cellular viability decreases. In particular, (Hussain, et al., 2005) suggested that silver nanoparticle-exposed cells became abnormal in size, displayed cellular shrinkage, and acquired an irregular shape. The authors concluded that exposure to this metal nanoparticle system resulted in cellular oxidative stress (Hussain et al., 2005). Other studies have shown that metal nanoparticles cause depletion of glutathione, reduced mitochondrial function and metabolic activity, and increased accumulation of metal ions (Braydich-Stolle et al., 2005; Hussain et al., 2005; Chen et al., 2006; Hussain et al., 2006; Meng et al., 2007; Guo et al., 2008; Lei et al., 2008; Midander et al., 2009). All of these toxicological observations at the cellular level are indicative of metal ion exposures and subsequent induced toxicities (Stohs and Bagchi, 1995; Cortese-Krott et al., 2009).

Here, we designed a study that incorporated a systematic approach to prepare and characterize the cellular responses after exposure to three different nano-sized metal colloids (nano-Ag, nano-Cu, and nano-Ni) over a dose-response and time-course range. Eventually, these data will aid in the prediction of nanomaterial-induced cellular responses using the physicochemical properties of the material.

Liver toxicity plays a vital role in drug and toxicant metabolism. Here, we used normal mouse hepatocytes, which represent a suitable model for

characterizing cellular susceptibility to metal toxicity (Safe, 1994; Vial and Descotes, 1994; Malik et al., 2000; Jaeschke et al., 2002; Xie and Tian, 2006). The liver is one of the most relevant tissues for nanomaterial toxicity from intentional medical or cosmetic applications or unintentional occupational exposures. While the route of entry for cosmetic application is the dermis and the route of entry for occupational exposures is the lung, ultimately, the particulate matter that is internalized (in the body) will be metabolized and excreted via the liver. Therefore, there is a need to understand nanoparticle properties and their effects in the body during the excretion process (Wan et al., 1991; Pinheiro et al., 1996; Das and Buchner, 2007). Potential nanoparticle-containing drugs or toxicants will eventually interact with liver tissue during excretion processes. Therefore, understanding nanomaterial-induced liver toxicity is pertinent to the successful development of metal-based nanotechnologies.

Here in this study we have used AML12 normal murine epithelial liver cells which provide a simple and physiologically relevant system. Co-culturing of parenchymal hepatocytes with non-parenchymal liver cells (NPC) can enhance liver function. The co-culture cell system can be used as bioartificial liver. To resolve some of the outstanding toxicity concerns, we need a model that more accurately mimics the response of the liver in an *ex vivo* cell culture system. Co-cultures of primary hepatocytes and NPC can be used to achieve that (Zinchenko, et al., 2006).

Although previously published research has shown that cells exposed to metal and metal oxide nanoparticles exhibit oxidative stress. The exact mechanism relating the oxidative stress and nanoparticle exposure is not well understood. Several literature reviews showed that nanoparticles can readily translocate across the membrane of many different cell-types (Limbach et al., 2007; Park et al., 2008). It has been shown earlier that cells exposed to several metal-containing nanoparticles can develop oxidative stress. Oxidative stress can be quantified by measuring the production of reactive oxygen species (ROS) by either the surface of the nanoparticle or by the mitochondria reacting to stress. An excess of these reactive molecules can cause cellular damage (Hussain et al., 2005; Park et al., 2008; Eom and Choi, 2009). As Limbach et al. has previously published, this oxidative stress can also occur through Trojan-horse type mechanism (Limbach et al., 2007).

Heat shock proteins (Hsp) have been utilized in determining metal toxicities and can be used to measure nano-sized metal toxicities. Hsp are molecular chaperones involved in protein folding and translocation (Bimston et al., 1998; Urani et al., 2001). Specifically, Hsp70 has been used as a general marker for cellular damage by metals (Urani et al., 2001). Arora et al. published a series of elegant studies that describe the dose-response relationships and induction of apoptosis after exposure to silver nanoparticles (Arora et al., 2008; Arora et al., 2009). Our goal was to expand on this work by comparing the dose-response and inflammatory responses of mammalian cells after exposure to

silver, copper, or nickel nanoparticles. Our study had two main objectives: first, to characterize and identify similarities in the physicochemical properties of nano-Ag, nano-Cu, and nano-Ni. The second objective was to determine the cytotoxicity and inflammatory responses of liver culture systems induced by the three metal colloids. Based on these results and other ongoing experiments in our laboratory, nanoparticle-induced toxicity and production of inflammatory biomarkers (on a cellular level) is found to be dependent on size, aggregation state, surface charge, and redox chemistry on the nanoparticle surface.

2.2 Materials and methods

2.2.1 Preparation and characterization of nano-sized metal colloids

Commercially manufactured nano-Ag, nano-Cu, and nano-Ni nanomaterials (approx. 30 nm) were purchased at 99.5% purity from Sigma-Aldrich (St. Louis, MO). The stock solutions of each material were prepared in Milli-Q ultrapure water. Each stock was then washed in phosphate-buffered saline (PBS) and analyzed in cell culture media (Sayes et al., 2007). Suspensions were serially diluted from the stock, resulting in concentrations from 0.1 to 1000 mg/L. Surface area was measured in the dry state using the BET method under N_2 absorption on a Micrometrics ASAP 2020 instrument (Brunauer et al., 1938; Reddy et al., 2001; Chen et al., 2004). TEM (JEOL 2010, JEOL Ltd., Tokyo, Japan) operated at 200 kV was used to determine agglomerate size and particle morphology (Wang, 2000). TEM grids were prepared by adding 2 µL of nanomaterial suspended in ethanol (100 ppm) onto

the copper grid surface (carbon-coated 400 mesh). Dynamic light scattering (DLS, Malvern Zetasizer Nano S 1600) was used to measure particle size populations and zeta potentials while in the aqueous suspension (Berne and Pecora, 1974; Panacek et al., 2006).

2.2.2 Cell culture conditions

Murine liver cells AML12 were purchased from American Type Tissue Collection (Manassas, VA**).** Liver cell cultures were plated at equivalent cell densities and exposed to the nanomaterial suspensions at 80% confluency. Cells were exposed to nano-Ag, nano-Cu, or nano-Ni suspensions and probed for cytotoxicity and inflammatory endpoints at 4 h, 24 h, 48 hr, and 1 week. For the 1-week time point, cell culture media was exchanged and re-dosed every other day. Exposure concentrations included 0.1mg/L, 1.0mg/L, 10mg/L, 100mg/L, and 1000mg/L concentrations of nanomaterials.

2.2.3 Isolation and culture of Kupffer's cells and lymphocytes

C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) were euthanized using CO_2 inhalation. Animals were provided humane care according to the institutional guidelines (University Laboratory Animal Care Committee, Texas A&M University). Following removal from the abdominal cavity, the liver was perfused with 30 mL of ice-cold serum-free RPMI 1640 using a peristaltic pump. Cells were collected in sterile centrifuge tubes and centrifuged at 300 rpm for 3 min. The pellet contained the majority of the hepatocytes, while most lymphocytes remained in the supernatant. The sample was decanted and

centrifuged again for 10 min at 1200 rpm. Both pellets (Kupffer's cells and lymphocytes) were then resuspended in serum-free RPMI 1640 medium (Crispe and Mehal, 1996; Mehal et al., 1999).

Both single cell-type cultures and co-cultures were maintained at 37°C with 5% CO_2 in Dulbecco's Modification of Eagle's Medium: Ham's F12K Medium (DMEM:F12K, American Type Tissue Collection (ATCC), Manassas, VA) and were supplemented with 10% fetal bovine serum (FBS, ATCC, Manassas, VA) and 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO) (Wu et al., 1994; Dumenco et al., 1995).

2.2.4 Analysis of cellular uptake of nanomaterials

For elemental analyses, inductively coupled plasma-mass spectroscopy (ICP-MS, Perkin Elmer DRC 2 spectrometer) was used to determine the identity and quantity of free metal ions released into the suspensions over time. Samples for ICP-MS were diluted and mildly digested using 5% hydrochloric acid and 1% nitric acid by volume (Becker, 2002; Guo et al., 2009). Differential interference contrast optical microscopy was also used to evaluate cellular uptake of nanomaterials using a Zeiss Stallion DDI workstation with a 63X, C-Apo 1.2 NA water immersion lens. Optical slices through cells (Z-stacks) were recorded in 0.5-μm steps to verify that nanomaterials were on the same plane as cytoplasmic organelles. Small aggregates of the nanomaterials exhibited movement in the cytoplasm that resembled cytoplasmic organelles, whereas those agglomerates adsorbed to the plasma membrane were static.

2.2.5. Cytotoxicity assessment

Cellular viability was measured using three independent techniques. First, the colorimetric dye, resazurin (Sigma Aldrich, St. Louis, MO) was used to determine the number of viable healthy cells as a function of the metabolism of resazurin to its metabolite resorufin (King et al., 1984; Putnam et al., 2002). Briefly, after the cultures were incubated with the nanomaterials for 4 h, 48 h, and 1 week, cells were removed from the incubator, media was aspirated, and the resazurin dye solution was added (10% of original cell culture media). After a 4 h incubation at 37°C, the samples were gently mixed in a gyratory shaker, and the resultant adduct formation was measured spectrophotometrically at a wavelength of 600 nm (Dutka et al., 1983). Second, the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan was used as an indicator of metabolic activity and to validate cytotoxicity results (Mosmann, 1983) (data not shown). Third, cell counts were measured using a Coulter Z1 particle counter (Chadalapaka et al., 2008).

2.2.6 mRNA extraction and RT-PCR analysis of cytokine and stress protein levels

The interference of nano-sized metal colloids on the production of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and heat shock protein 70 (Hsp70) mRNA was evaluated. Total RNA was extracted from the nanomaterial-exposed liver cell culture systems using the RNeasy Midi kit (Quiagen, Valencia, CA) and used for one-step real-time quantitative

polymerase chain reaction (RT-PCR) via SYBR Green (Apte et al., 2005). An ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) was used to denature the samples at 95°C for 10 min. The thermal cycling step was for 40 cycles at 95°C for 15 s, and 40 cycles at 60°C for 1 min. The thermal cycling was followed by the dissociation step (95°C for 15 s, at 60°C for 1 min, and then 95°C for 15 s). Each reaction mixture contained both forward and reverse primers of IL-6 and TNF-α, RNase inhibitor, and Reverse Transcriptase in a SYBR Green master mix (Applied Biosystems, Foster City, CA). Primers were obtained from IDT (Coralville, IA) and used for amplification. The software Primer Express (Applied Biosystems, Foster City, CA) was used to design the primers: IL-6 (Forward: 5'-AGT TGC CTT CTT GGG ACT GA-3'; Reverse: 5'-TCC ACG ATT TCC CAG AGA AC- 3'). TNF-α (Forward: 5'- AGC CCC CAG TCT GTA TCC TT-3'; Reverse: 5'-CTC CCT TTG CAG AAC TCA GG-3′). Hsp70 (Forward: 5′-CGA GGC TGA CAA GAA GAA-3′; Reverse: GGC CTC TAA TCC ACC TCC TC). The mRNA levels were normalized using β-actin (Forward: 5'-CCG TGA AAA GAT GAC CCA GAT-3'; Reverse: 5'-CAC AGC CTG GAT GGC TAC GT-3') as an internal housekeeping gene (Chintharlapalli et al., 2007).

2.2.7 Probe for reactive oxygen species, in vitro

Reactive oxygen species (ROS) production in the nanomaterial-dosed cells was examined intracellularly using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma, St. Louis, MO. USA) (AshaRani et al., 2009). Briefly, colloid-dosed liver cells were washed with PBS and incubated with DCFH-DA for

30 mins. The fluorescence of each nanomaterial-exposed sample was measured at ex/em 485/520 nm. (Hussain et al., 2005; Park et al., 2008; Eom and Choi, 2009).

2.2.8 Western blot analysis of Hsp70

Hepatocytes were plated as described above. Cells were washed with ice-cold PBS and lysed with high-salt lysis buffer. Lysates were spun at 12,000 x g for 15 min at 4°C, and supernatants were collected. The amount of protein present was determined using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, Del.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 8% protein gels was used to quantify expression of Hsp70. After running the gel, the samples were transferred into nitrocellulose membrane. The membranes were blocked with 0.1% Tween and 6% non fat powdered milk in PBS buffer for 3 h (Apte et al., 2005), followed by overnight incubation with Hsp70 rabbit polyclonal primary antibody (Assay Designs, Inc, Ann Arbor, MI). The membranes were then washed with PBS and 0.1% Tween and incubated in HRP-conjugated anti-rabbit secondary antibody. β-actin was used as an internal control to ensure equal loading of proteins per well. The membranes were then washed with TBST for 10 min, incubated with chemiluminiscence reagent from Perkin-Elmer for 1 min and exposed using Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY) (Crosby et al., 2000; Apte et al., 2005; Chintharlapalli et al., 2007).

2.2.9 Statistical analyses

Each experimental value was compared to the corresponding control value for each time point. One-way ANOVA and Bartlett's tests were calculated for each sampling time. When the F-test from ANOVA was significant, the Dunnett's or Dunn's test was used to compare means from the control group and each of the groups exposed to colloids. Statistical significance of exposed cell populations versus unexposed control populations was established as $p<0.05$. Statistical tests were performed with SAS software (SAS Institute Inc, Cary, NC) (Sayes et al., 2007).

2.3 Results and discussion

2.3.1 Nano-sized metal colloid characterization

The first step in evaluating nano-sized metal colloid toxicity was focused on understanding the physical properties, including surface charge and agglomeration dynamics of nano-sized metal colloidal systems. Fig. 1 shows DLS sizing patterns and indicating that nano-Ag exhibited a bimodal sizing profile with peaks at 30 nm and 122 nm. The size distribution curves did not change over time (up to 1 week after preparation of nanomaterial suspensions). In contrast, nano-Ni exhibited varying bimodal and, at some time points, polymodal sizing profiles over the 0 h to 1 week time period. The particles seemed to form agglomerates and then to de-agglomerate throughout the analysis periods. Nano-Cu exhibited similar behavior compared to nano-Ag in that the sizing profiles remained static from 0 h to 12 h post-exposure. At 48 h,

the sizing peak shifted slightly to the right, indicating the formation of larger agglomerates. An even larger shift to the right was observed at the 1 week time period. Nano-Cu differed from nano-Ag in that the nano-Cu DLS pattern showed one peak, where the nano-Ag DLS pattern showed two peaks.

TEM provided information on the primary particle size (Fig. 10). All three particle types had a primary particle size distribution ranging from approximately 15 nm to 70 nm, with an average particle size of 30 nm. Micrographs of nano-Ag particles showed faceted sides, but were irregularly shaped; nano-Ni particles were spherical; and nano-Cu particles were irregularly shaped but had ill-defined surfaces.

Surface area was measured based on the density of the metal and used to calculate particle size using the formula: $D = 6/ (d \times A)$, where D is particle average diameter, d is particle density, and A is the measured surface area (Sayes et al., 2007). For nano-Ag, the surface area was 4.9 m^2/g, the density was 10.5 g/cm^3 and the resultant calculated size was 116 nm. For nano-Ni, the surface area was 4.7 m^2/g, the density was 8.9 g/cm^3, and the calculated size was 143 nm. For the nano-Cu, the surface area of 9.9 m^2/g, the density was 8.9 g/cm^3, and the calculated size was 68.1 nm (Table 1).

Both nanomaterial agglomeration state and cellular uptake are influenced by the surface charge of the nanomaterial. Table 1 also reports the differential surface charge of nano-Ag, nano-Ni, and nano-Cu at 4 h, 48 h, and 1 week. Zeta potential measurements showed a similar pattern for all three nano-sized metal

colloidal systems over the 1-week time period, i.e. the zeta potential value changed from a more negatively charge to a less negative charge. Nano-Ag changed from -45 to -36 to -30 mV; nano-Ni changed from -25 to -8 to 0 mV; and nano-Cu changed from -29 to -17 to -15 mV.

Table 1.
Physical properties of the three metal colloidal systems used in this study. The table compares the surface area, density, calculated primary particle size, measured hydrodynamic diameter at t=0, t=48 h and t=1 week, and zeta potential at t=0, t=48 h and t=1 week

Property	Ag	Ni	Cu
Measured size from DLS @ t=4h (nm)	122.4	122.4	255
Measured size from DLS @ t=48h (nm)	122.4	1990	396.1
Measured size from DLS @ t=1week (nm)	122.4	190.1	1106
Zeta potential @ t=4 h(mv)	-45.2	-25.1	-29.1
Zeta potential @ t=48 h (mv)	-36.5	-8	-17.8
Zeta potential @t=1 week (mv)	-30.7	-0.8	-15.7

Considering particle size and surface charge observations, it was hypothesized that charged particle surfaces in aqueous suspensions helps to maintain stable nanoagglomerates (i.e. nano-Ag) over time. Nano-Cu and nano-

Ni particle suspensions demonstrated loss of surface charge and variation of aggregate size.

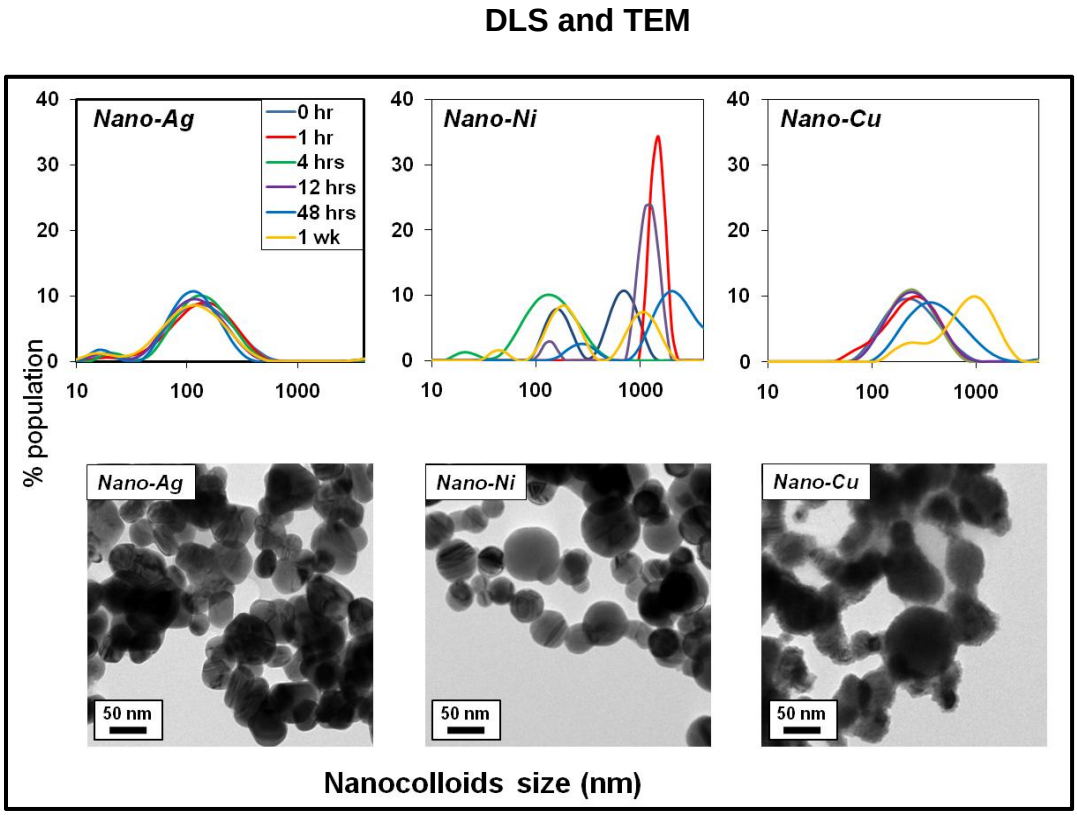

Fig. 10. Sizing profiles of nano-Ag, nano-Ni, and nano-Cu. The dynamic light scatter plots of each of the three metals are compared with their corresponding transmission electron micrographs.

2.3.2 Cellular uptake of nano-sized metal colloids

Two independent methods were used to verify nanomaterial cellular uptake: inductively coupled plasma-mass spectroscopy (ICP-MS) and optical microscopy. After exposure to each of the nano-sized metal colloids, independently, ICP-MS was used to determine the amount of metal (Ag, Ni, or

Cu) in parts per million (ppm) concentrations within the spent media, the phosphate buffered saline (PBS) solution wash, and the cell lysates. Optical microscopy showed evidence of both large and smaller agglomerates inside the cell. Taken together, the data indicated that the colloids were taken into the cells. However, the exact cellular uptake mechanisms are not well understood. ICP-MS analysis demonstrated that all three nanomaterials were taken up into the cell as indicated by the concentration of metal in the cell lysates. However, there were differing amounts of metal in each sample. The amount of silver in nano-Ag exposed cell lysates was 1.6 ppm, the amount of copper in nano-Cu exposed cell lysates was 0.123 ppm, and the amount of nickel in nano-Ni exposed cell lysates was 4.61 ppm. For cells exposed to nano-Ag and nano-Ni, most of the metal is in the cell lysates. But for cells exposed to nano-Cu, most of the metal is in the spent media. The optical micrographs indicate the same phenomenon. Both small and large agglomerates were seen inside the cells exposed to nano-Ag and nano-Ni, but no agglomerates were seen inside the cells exposed to nano-Cu. Fig. 11 also includes ICP-MS analyses on two control suspensions. First, the cell culture media was analyzed for residual amounts of silver, copper, and nickel. The bars in the graphs are labeled as “media”. Second, the cell culture media spiked with a 35% v/v suspension of 100 ppm nano-Ag, nano-Cu, or nano-Ni was analyzed for silver, copper, and nickel.These bars are labeled as “Ag spiked media,” “Cu spiked media,” and “Ni spiked media.”

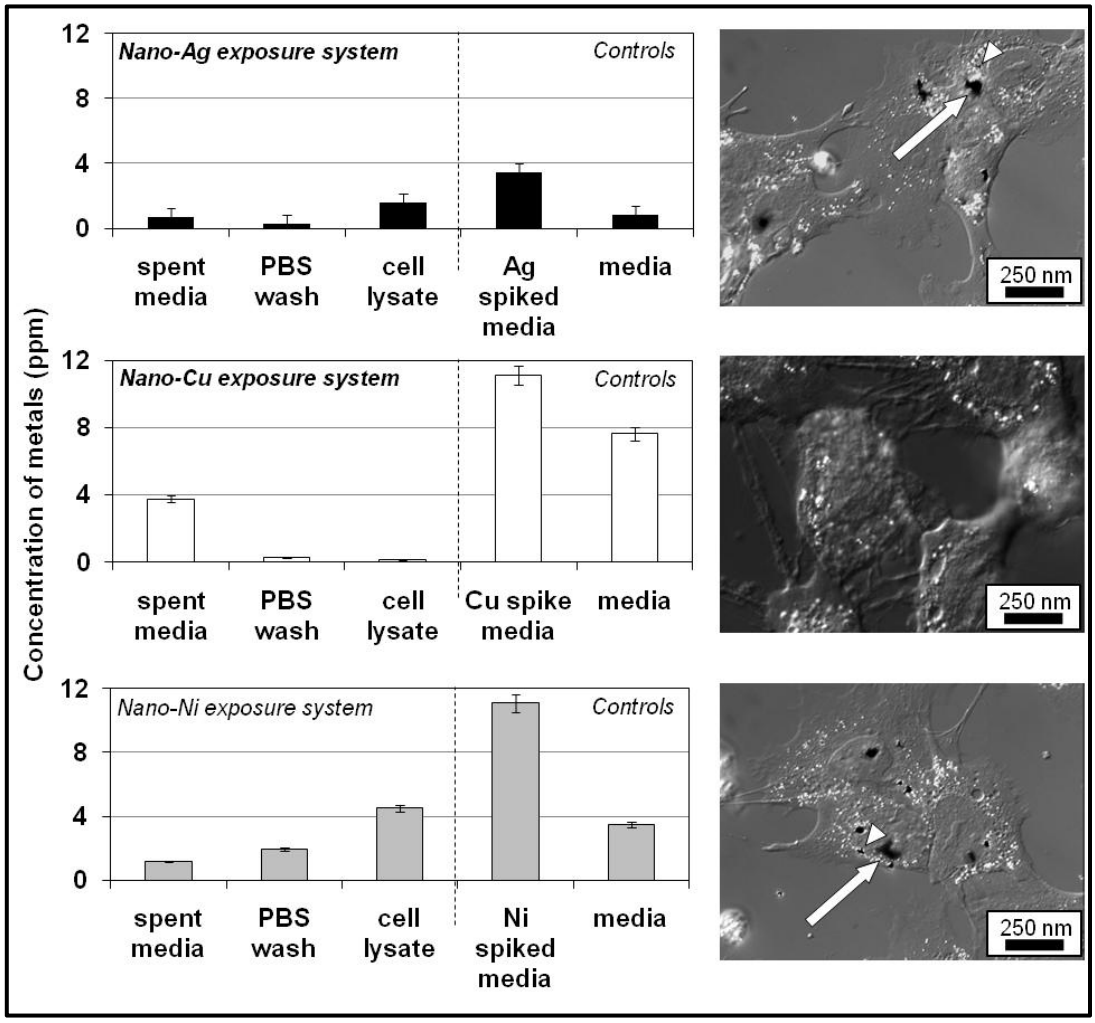

Fig 11. Evidence of nano-metal colloid cellular uptake. Endocytosis of nano-Ag, nano-Cu, and nano-Ni was verified using two independent methods: inductively coupled plasma-mass spectroscopy and optical microscopy. AML12 cells were inoculated with the different nano-sized metal colloids. All doses are 350 µL of 100 mg/L. Images were taken at 36 h post-exposure. Control suspensions include the pristine cell culture media (labeled as “media”) and the cell culture media spiked with 35% v/v suspension of 100 ppm nano-Ag, nano-Cu, or nano-Ni (labeled as “Ag spiked media”, “Cu spiked media”, and “Ni spiked media”). Each suspension was analyzed for silver, copper, and nickel using ICP-MS.

2.3.3 Cytotoxicity and inflammatory responses

Liver culture cell viability and production of inflammatory cytokine biomarkers and stress protein mRNA expression are shown in Fig. 12 to Fig 16. For the nano-Ag exposed single cell culture system and co- culture system, no significant cell death was observed at the 4 h and 48 h exposure time points. Significant decrease in viability was observed at the 1 week time point, relative to control cell culture systems. For the nano-Cu exposed single cells culture system and co-culture system, significant cell death was observed at all exposure time points (Fig. 12 and Fig. 13). For the nano-Ni exposed cells, significant cell death was observed at the 4 h and 1 week exposures whereas for the nano-Ni exposed co-culture system, no significant cell death was observed (Fig. and Fig. 13). Further, there was a differential decrease in viability among the three nanomaterial systems, where nano-Cu decreased viability by 30% at the 0.01 mg/L dose (as measured by the metabolism of resazurin dye), nano-Ni decreased viability by 25% at the 0.01 mg/L dose, and nano-Ag decreased viability by 5% at the 0.01 mg/L dose (Fig.). Results indicated similar trends using the MTT assay for metabolic activity: metabolic activity was decreased the most after exposure to nano-Cu, metabolic activity was slight decreased after exposure to nano-Ni, and metabolic activity did not decrease, relative to control cells, after exposure to nano-Ag (data not shown)**.**

In contrast to cellular viability, when measured inflammatory cytokine biomarker differential responses between the single cell culture system versus

the co-culture system, similar results were observed for the two test systems. Exposure to nano-Ag did not produce significant increases in TNF-α, IL-6, or Hsp70 levels, while exposures to nano-Cu and nano-Ni produced significant inflammatory responses (Fig. 14 and Fig. 15) and increased mRNA level of stress protein Hsp70 in liver cell line (Fig. 16).

Generally, toxicological studies show linear or exponential increase in response as the dose is increased. But, in nanotoxicology studies, because there is a limit to the increase in nanoparticle dose, there is also a limit to the increase in nanoparticle relevant response. In the results included in this study, as with many data sets published in the literature, nanotoxicity data sets show that there is a drop in response after a relatively large nanoparticle dose. This effect is probably due to the fact that nanoparticles aggregate or agglomerate and do not act as nanoparticles at higher concentrations.

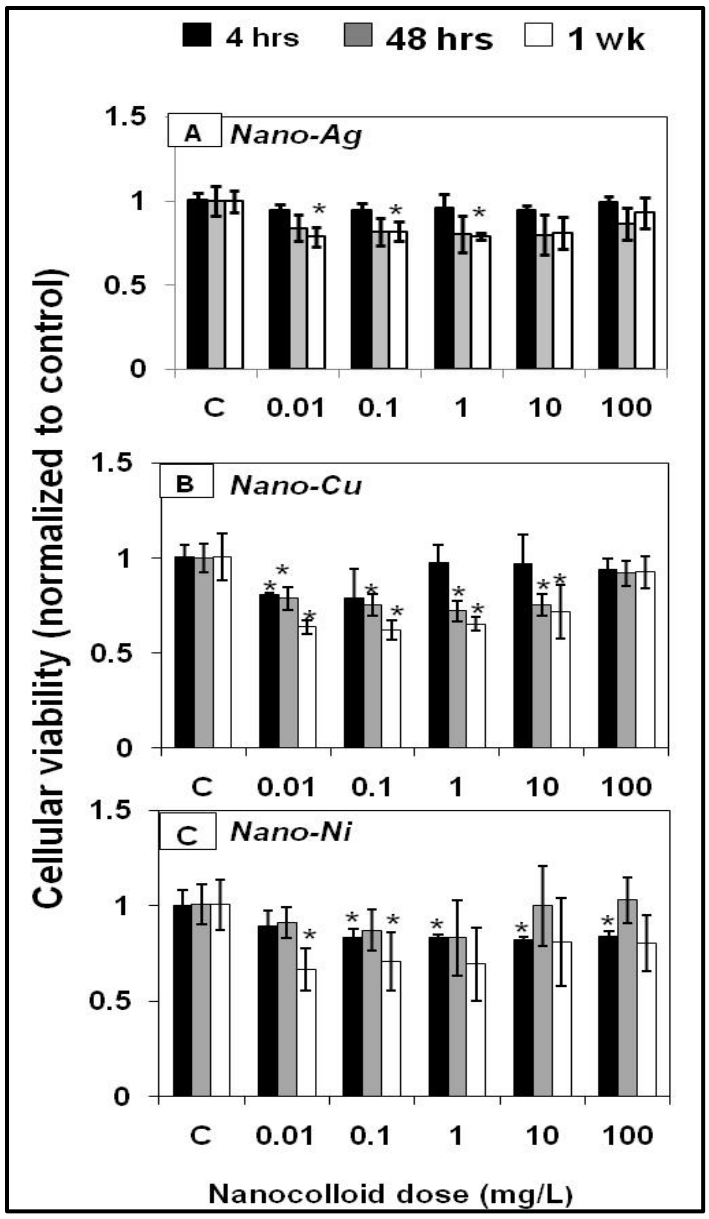

Fig. 12. Effect of nano-sized metal colloids on cellular viability in AML12 mice heptocyte cultures. Cells were treated with increasing concentrations of nano-Ag, nano-Cu, or nano-Ni for 4 h 48 h, and 1 week. (A) Cell cultures were inoculated with nano-Ag, (B) cell cultures inoculated with nano-Cu, (C) cell cultures inoculated with nano-Ni. At the end of the incubation period, metabolic activity was determined by the production of the metabolite resorufin. Absorbance was measured at 595nm. Background absorbance of multiwell plates and of nanomaterial spiked cell culture media was subtracted from measurement. *$p < 0.05$ vs. control cell population

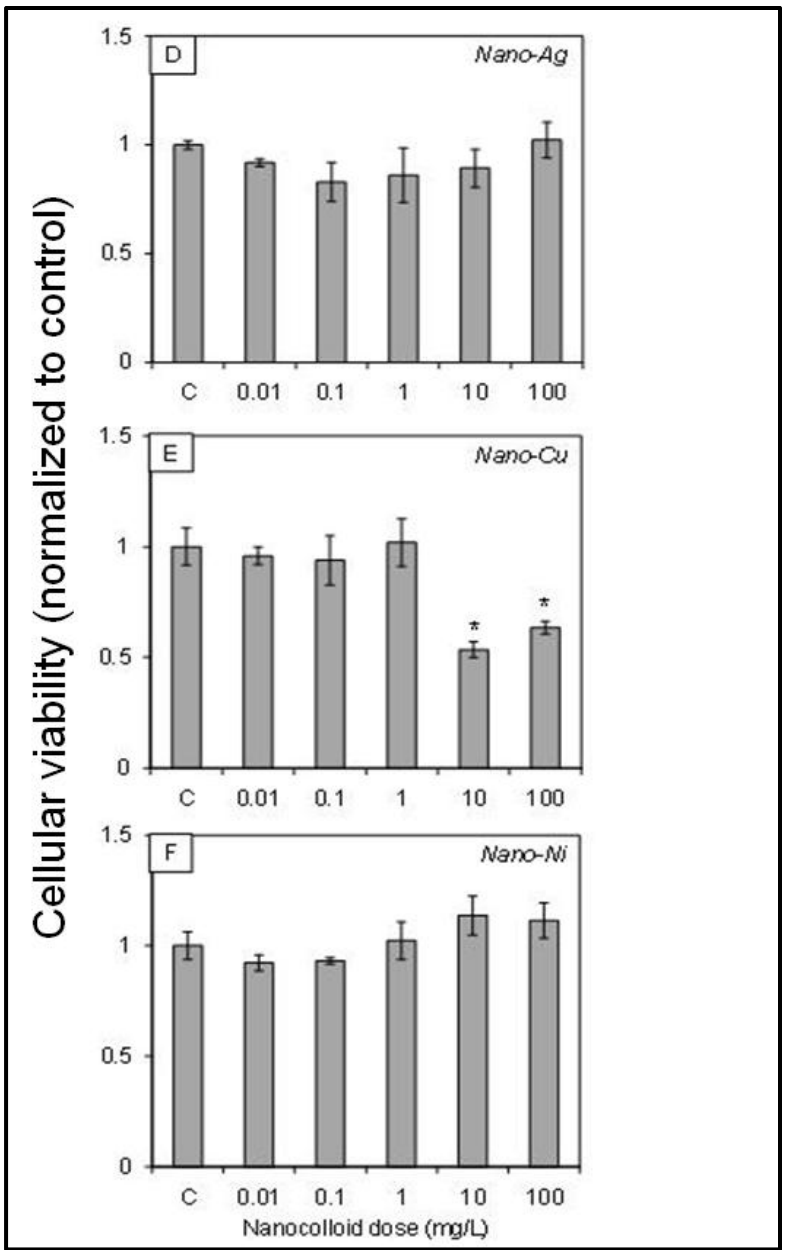

Fig. 13. Effect of nano-sized metal colloids on cellular viability in co-culture of primary Kupffer's cells, lymphocytes isolated from C57BL6 mice and AML12 mice heptocyte cultures. Cells were treated with increasing concentrations of nano-Ag, nano-Cu, or nano-Ni for 48 h. (D) Cell cultures were inoculated with nano-Ag, (E) cell cultures inoculated with nano-Cu, (F) cell cultures inoculated with nano-Ni. At the end of the incubation period, metabolic activity was determined by the production of the metabolite resorufin. Absorbance was measured at 595nm. Background absorbance of multiwall plates and of nanomaterial spiked cell culture media was subtracted from measurement. *$p < 0.05$ vs. control cell population

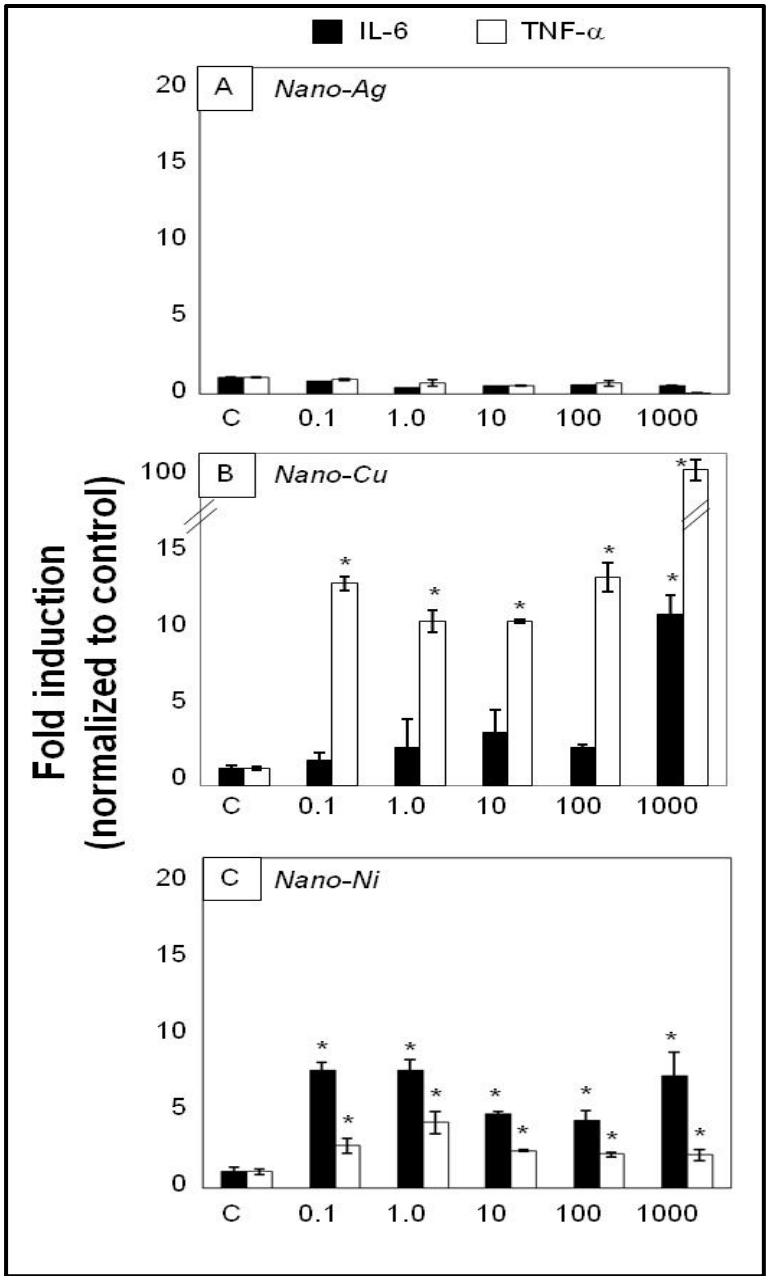

Fig. 14. Analyses of cytokine messenger RNA levels in AML12. TNF-α and IL-6 mRNA detection after exposure to nano-colloid metals by RT-PCR from AML12 hepatocyte cultures. (A) Single cell cultures were inoculated with nano-Ag, (B) single cell cultures inoculated with nano-Cu, (C) single cell cultures inoculated with nano-Ni. The values were normalized with beta actin, the housekeeping gene. *p < 0.05 vs. control cell population.

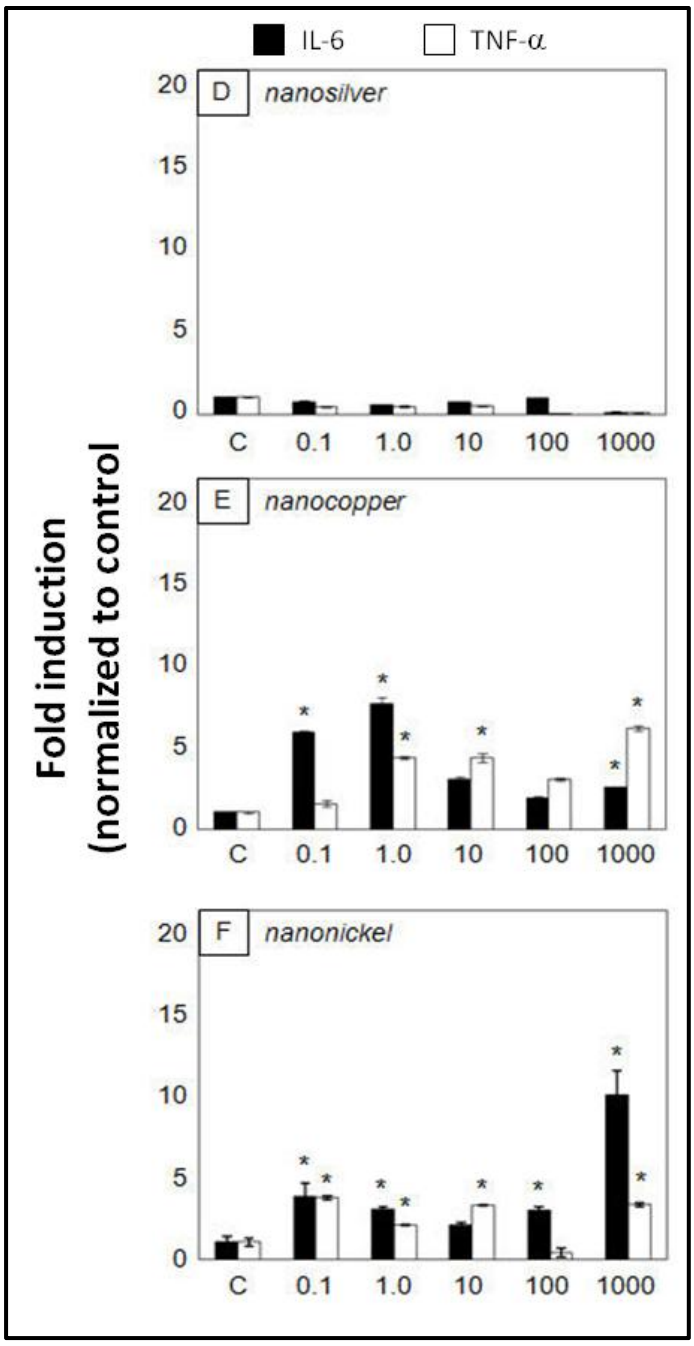

Fig. 15. Analyses of cytokine messenger RNA levels in co-culture. TNF-α and IL-6 mRNA detection after exposure to nano-colloid metals by RT-PCR from co-culture of primary Kupffer's cells, lymphocytes isolated from C57BL6 mice and AML12 mice heptocyte cultures (D) Co- culture cells were inoculated with nano-Ag, (E) Co-culture cells were inoculated with nano-Cu, (F) co-culture cells were inoculated with nano-Ni. The values were normalized with beta actin, the housekeeping gene. *$p < 0.05$ vs. control cell population.

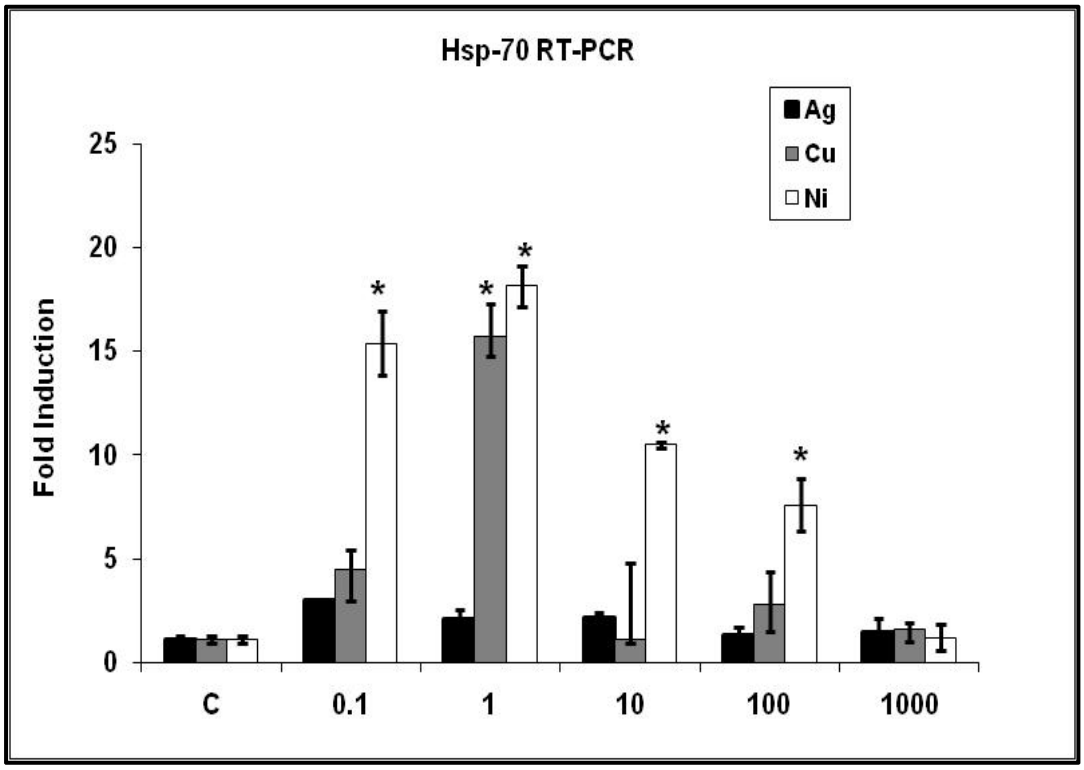

Fig. 16. Hsp70 mRNA expression by RT-PCR from AML12 hepatocyte cultures. (A) Single cell cultures were inoculated with nano-Ag, (B) single cell cultures inoculated with nano-Cu, (C) single cell cultures inoculated with nano-Ni. The values were normalized with beta actin. *$p < 0.05$ vs. control cell population.

2.3.4 Cellular stress responses

Results from western blot analyses of stress protein biomarker expression (hsp70), after exposure to nano-Cu, nano-Ni and nano-Ag, showed similar trends to the IL-6, TNF-α, and cytotoxicity data sets. Nano-Cu and nano-Ni increased hsp70 expression; however, no significant changes were observed after exposure to nano-Ag (Fig. 17).

The enhanced expression of hsp70 after exposure to nano-Cu and nano-Ni provided a protection against activation of heat shock factor caused by the heavy metals. The mechanism behind these observations is not well understood. However, we propose that after exposure to copper and nickel nanoparticles, dissociated free metal ions from the particle surface (via the intracellular acidification process) enhance expression of hsp70 at sublethal concentrations. The increased expression of Hsp70 gene coding for the protein after exposure to copper salts has been already shown in many other cell types (Urani et al., 2001).

2.3.5 Production of reactive oxygen species by nano-Ag, nano-Cu and nano-Ni

The cellular ability to produce ROS was measured by dichlorofluorescin diacetate (DCFH-DA) fluorescence. DCFH-DA was hydrolyzed to form dichlorofluorescin (DCFH), which remained within the cell. This molecule was then oxidized to fluorescent dichlorofluorescin (DCF). Here, DCF fluorescence, in copper and nickel exposed cells, increased in a dose-dependent manner as compared to the control cell and silver exposed cell populations (Fig.18), showed that intracellular ROS production increased in cultures exposed to 100 and 1000 ppm of nano-Cu and 10, 100, and 1000 ppm of nano-Ni.

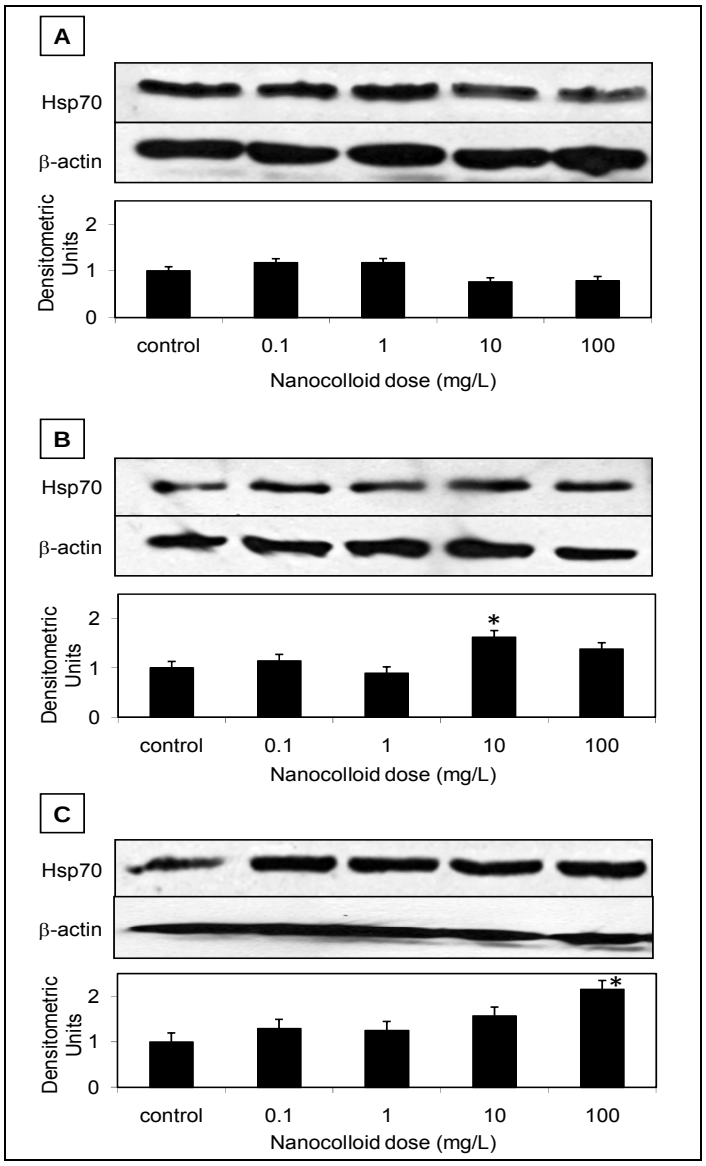

Fig. 17. Hsp70 protein expression by Western blot analyses in AML12 cells dosed with (A) nano-Ag, (B) nano-Ni, and (C) nano-Cu. Graphs below each western blot indicates densitometric analysis. β-actin was used as an internal calibration for these analyses to ensure equivalent protein loading. *$p < 0.05$ vs. control cell population.

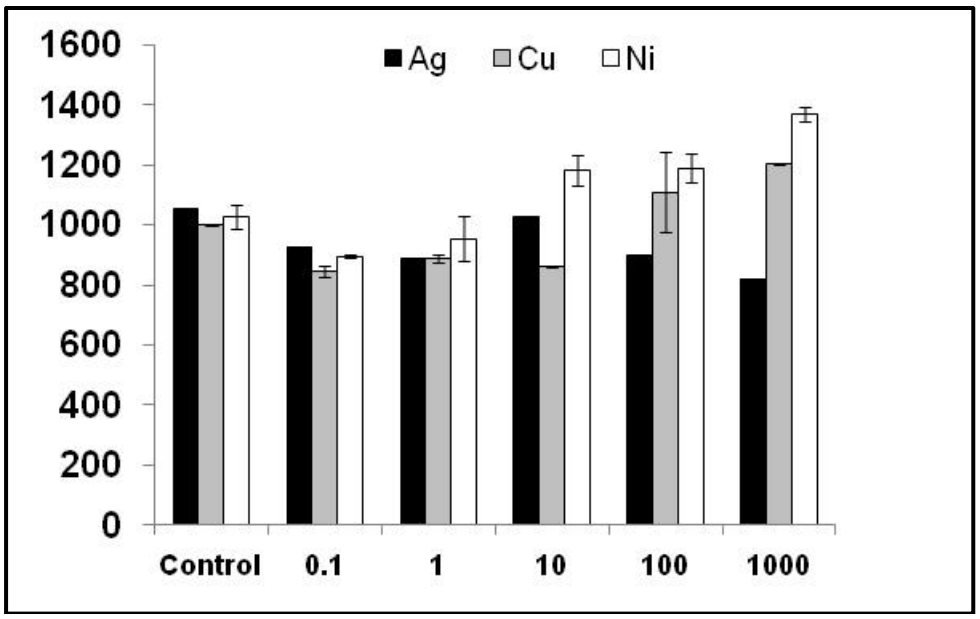

Fig. 18. Production of ROS in AML12 cells by nanomaterials. The cells were dosed with nano-Ag, nano-Cu and nano-Ni for 24 h and of 40 μm of DCFH-DA at 37°C for 30 min. The fluorescence in cells was observed using a fluorescence plate reader.

3. SUMMARY

Here, we give an explanation for the differential inflammatory response of hepatocytes after exposure to nano-Ag, nano-Cu, or nano-Ni. The critical steps in nano-sized metal colloid toxicity could include: (1) nanoparticle uptake within the cell, (2) nanoparticle releasing free metal ion or dissolution of metal ions from the nanomaterial core, and (3) increase in pro-inflammatory cytokinemRNA levels. After exposure to a suspension of nano-sized metal colloids, cells in culture internalized the particles intracellularly as either small nanoagglomerates (90-150 nm) or larger agglomerates (200-500 nm). Silver, copper, or nickel ions are leeched from the surface of these agglomerates, either in the cell culture media before internalized or within acidified vesicle. The magnitude of charge on the surface of the nanoagglomerates is inversely related to the concentration of free metal ions in the cell culture media. The concentration of free ions in the culture medium contributes to the decrease in cellular viability and induction of inflammatory cytokines.

The inflammatory response of hepatocytes exposed to nano-Cu is not due to the nanoparticle exposure alone, but due to a combination of the slight cellular uptake of individual nanoparticles (not nanoagglomerates) and solubilized copper ions in the culture media. Exposure to copper ions from food, through skin or eye contact, or by inhalation of dusts can lead to liver damage (Lei et al., 2008). Furthermore, recent studies suggest that a genetic defect is associated with copper poisoning (Johnson, 2008). Intake of copper salts has

produced acute copper toxicity in humans and equivalent amounts of copper salts are toxic in animals. Ingestion by animals of three ounces of a 1% solution of copper ions will produce inflammation of the gastrointestinal tract. When copper salts are administered intravenously, as little as 2 mg/kg copper ions are lethal to guinea pigs and 4 mg/kg are lethal to rabbits (TOXNET, 1975-1986).

Exposure to nano-Ag did not decrease cellular viability, nor did nano-Ag exposure induce an inflammatory response, supporting earlier observations (Ward and Saffle, 1995; Warila et al., 2001; Braydich-Stolle et al., 2005; Harper et al., 2006; Singh et al., 2007; Suzuki et al., 2007). Some have speculated that because silver does not have a +2 valence state, it does not oxidize into a toxic form. We hypothesize that the negatively charged surface on nano-Ag particles stabilizes the particle and prohibits ion leeching in cell culture media. Further data is needed to address this hypothesis directly.

We have previously published a mechanism for inducing oxidative stress after nanomaterial exposure via acidified cell organelle's solubilizing water-insoluble metal oxide particles and hence producing free metal ions (Guo et al., 2009). In that report, Fe_3O_4 nanomaterials that released Fe^{+3} once inside acidifed vesicles did not induce oxidative stress. Instead, it was the reductive capacity of the engineered carbon black material that reduced Fe^{+3} to Fe^{+2}, resulting in oxidative stress. In the present study, it was determined that cellular toxicity was, in part, due to the production of Ni^{+2} and Cu^{+2} ions from nano-Ni and nano-Cu surfaces in the culture media and/or inside of the cell. However, a

combination of nanomaterial physicochemical properties might exist that either increases or decreases their biological impact.

The present study investigated the induction of inflammatory response to ROS-producing nano-sized metal colloids. The surface properties, specifically the increasingly neutral surface charge of nano-Cu and nano-Ni, are hypothesized to be influential factors in nanoparticle-induced cytotoxicity, production of inflammatory cytokines, and may be responsible for the intracellularly produced reactive oxygen species. In contrast, nano-Ag maintains a large negative surface charge over time and little intracellular production of ROS is observed in dosed cells.

In conclusion, results showed predicting nanomaterial toxicities based on identifying trends in physical and chemical properties has the potential to aid in the development of nanomaterial environmental health and safety assessments. In these studies, the following relations were established: nano-Cu particles formed small agglomerates, maintained little surface charge, induced inflammation, but did not decrease cellular viability; nano-Ni formed large agglomerates, lost its surface charge, induced inflammation and decreased cellular viability; and nano-Ag particles remained individual particles, also formed small agglomerates, maintained charged surfaces, and did not induce inflammation or decrease cellular viability.

In summary, our studies show that nano-Ag, Nano-Cu and nano-Ni all the three nano- sized metal colloids internalize inside the liver cells. But among the

three different metal colloids, nano-Ag appeared to be less toxic with less reactive oxygen species (ROS) production and with little significant changes in the pro-inflammatory cytokine expression than the other two metal colloids.

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